高等植物の液胞タンパク質の成熟化機構に関する細胞生物学的研究

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学位申請論文

題目　高等植物の液胞タンパク質の成熟化機構に関する細胞生物学的研究

植物の種子は，次世代の胚が生長するための栄養源としてタンパク質を大量に蓄えている．このタンパク質は種子の形成過程中に合成され，細胞内の液胞に蓄積される．一般に植物の液胞は細胞内不要産物の分解の場と捉えられていることから考えると，この時期の種子細胞の液胞は極めて特殊な機能を持つといえる．当該研究室では，種子細胞の液胞に含まれる様々な種子タンパク質の生合成と細胞内輸送機構の解析を進めてきた．その結果，種子タンパク質がrERで前駆体として合成され，デンスベシクルによって液胞に送られることを示すと共に，液胞プロセシング酵素 (VPE) によってプロセシングを受け，液胞内に蓄積することを明らかにした．種子タンパク質をはじめとした液胞タンパク質の成熟化は，一連の液胞タンパク質の生合成過程の最終段階で起こることから，液胞タンパク質の最終的な構造を決定する重要な現象と見ることができる．この液胞タンパク質の成熟化機構を理解するため以下の研究を遂行した．

液胞プロセシング酵素 (VPE) の種子細胞内における局在及び様々な植物器官におけるVPE活性の検出

VPEはデンスベシクル中に含まれる種子タンパク質前駆体のプロセシングを行う酵素として見出された．ヒマ登熟種子細胞内のVPEの局在性について免疫電子顕微鏡観察及び細胞分画を行って調べたところ，VPEが液胞内の可溶性領域に局在していることが判り，種子細胞内の液胞で機能していること

が明らかになった．VPEは前駆体タンパク質のアスパラギン残基のC末端側のプロセシングを行うが，これと同様のプロセシングが本葉や根といった栄養器官の液胞タンパク質の成熟化の過程でも起こることが知られている．VPEが種子いわゆる貯蔵器官だけでなく栄養器官で機能しているかどうかを検討するため，合成ペプチドを基質とした活性測定を行ったところ，VPE活性が様々な植物の栄養器官から検出された（報文１）．最近，シロイヌナズナから３種類の本酵素のホモログの遺伝子が単離され，それらの発現様式についての解析から，貯蔵器官に局在する酵素群と栄養器官に局在する酵素群に分かれることが明らかになってきている．この結果も，貯蔵器官から見出されたVPEが栄養器官でも機能していることを支持している．

酵母を用いたVPEの活性発現機構についての解析

ヒマ種子の免疫電子顕微鏡観察によりVPEが液胞のみならずデンスベシクルにも局在することが判った．デンスベシクルは種子タンパク質前駆体を多量に蓄積しているが，単離したデンスベシクル内ではこれらの前駆体の成熟化は起こらない．この結果から，デンスベシクル内に局在するVPEは不活性型前駆体であると考えられた（報文１）．不活性型前駆体の存在はVPEのcDNAを用いた解析からも支持され，VPEの不活性型前駆体を活性型へ変換する活性発現が植物液胞内で生じることを示唆している．

VPEの活性発現機構について調べるため酵母細胞で本酵素の発現を行ったところ，酵母の液胞内で不活性なVPE前駆体が限定分解を受け，生じた発現産物がVPE活性を持つことが判った．酵母の液胞内プロテアーゼの１つであるカルボキシペプチダーゼY前駆体はアスパラギン残基のC末端側で切断を受け，成熟化することが知られている．酵母細胞内に発現させたVPEがこのカルボキシペプチダーゼY前駆体をもプロセスしたことから，酵母液胞内でもVPEが活性を持ち，機能していることが示された（報文２）．

VPEはそのcDNAを用いた解析からパパインに代表される既知のシステインプロテアーゼ群とは系統が異なる，新規のシステインプロテアーゼである．パパインの活性中心近傍のアミノ酸配列とVPEホモログ間のアミノ酸配列との比較から，ヒマVPEは開始メチオニンから83番目のシステイン残基及び

180番目のヒスチジン残基を活性中心に持つことが予想される．そこでこれらのアミノ酸残基をグリシン残基に変えた変異タンパク質を発現させたところ，VPE前駆体が蓄積するのみで限定分解が起こらず，VPE活性も検出されなかった．このことから，酵母で発現させたVPEが液胞内で自己限定分解により活性化されていることが示唆された．

これらの結果は，植物の液胞内へ輸送されてきたVPEの前駆体が自己触媒的に活性化され，この活性型酵素が各種の液胞タンパク質の成熟化に関与しているというカスケード機構の存在を強く示唆している．ここで成功した酵母細胞での発現系を用いた解析により，VPEによる植物液胞タンパク質の成熟化現象の生理的役割について明らかになることが期待される．

アスパラギン酸プロテアーゼ(AP)の液胞タンパク質の成熟化への関与

酵母ではプロテイナーゼAがカルボキシペプチダーゼYをはじめとする液胞タンパク質の成熟化に関与していると考えられている．プロテイナーゼAや動物のカテプシンDと類似性を示すアスパラギン酸プロテアーゼ (AP)が植物液胞タンパク質の成熟化に関与しているかどうかを検討するため，ヒマ種子から48 kDaのAPを精製した．精製酵素に対する抗体を調製し，その抗体を用いて細胞内の局在性及び種子の登熟期から発芽生長期における酵素量の変動を調べた．その結果，APはVPEと同様にヒマ種子細胞の液胞内に局在し，その酵素量が種子の登熟期には増加し，発芽生長期には減少することが判った．しかし精製VPEがデンスベシクル内の複数の前駆体タンパク質をプロセスするのに対して，精製APによっては，どのタンパク質のプロセシングも起こらなかった（報文3）．

種子タンパク質の１つである2Sアルブミンの前駆体は２つのアスパラギン残基のC末端側でVPEによるプロセシングを受けることにより，２つのサブユニットに分かれる．成熟型2Sアルブミンの一次構造と前駆体のアミノ酸配列とを比較すると，2Sアルブミン前駆体は成熟型タンパク質には存在しないプロペプチドを持つことが判る．2Sアルブミンの成熟化の過程で，VPEによるプロセシング後にこのようなプロペプチドの除去が必要となってくる．ここで精製したAPはシロイヌナズナ2Sアルブミンのプロペプチド断片を複数箇

所で切断する活性を持つことから，APがプロペプチドの分解除去を行っていると考えられた．以上のことから，液胞タンパク質前駆体は液胞内でVPEにより第一段階のプロセシングを受け，更にAPにより残るプロペプチドの分解が触媒されることによって，成熟型に変換されることが示唆された．

目　次

略　号

BPB	: bromophenol blue
BSA	: bovine serum albumin
CBB	: Coomassie Brilliant blue
cDNA	: complementary DNA
EDTA	: ethylenediamine tetraacetic acid
ER	: endoplasmic reticulum
HEPES	: N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid
IgG	: immnoglobulin G
kb	: kilobase
kDa	: kilodalton
mRNA	: messenger RNA
PBS	: phosphate-buffered saline
SDS	: sodium dodecyl sulfate
SDS-PAGE	: SDS-polyacrylamide gel electrophoresis
TBS	: Tris-buffered saline
Tris	: tris (hydroxymethyl) aminomethane

その他，核酸，アミノ酸の略号として一文字表記及び三文字表記をそれぞれ用いた．

序　論

高等植物の液胞は，動物のリソソームと対比できるとされ，多くの加水分解酵素を含むことが示されて以来，植物細胞中の不要産物の分解を行うオルガネラであると考えられてきている．しかし，実際は非常に多様な機能を担ったオルガネラであり，組織，器官や植物の成長段階によってその形態を大きく変動させる能力を備えている．中でも種子細胞の液胞は，タンパク質を多量に蓄積するという，分解とは全く逆の機能を登熟期にのみ発揮する．このことから種子細胞の液胞は高等植物液胞一般の機能を理解するための格好のモデル系を含んでいるといえる．以下に植物の種子細胞に見られる液胞の内部に存在する種子タンパク質や複数のプロテアーゼについて簡単に紹介していく．

種子の形成と種子タンパク質の蓄積

種子は次世代の胚のみならず，それが生長するための栄養源をも貯蔵する．この種子の形成と貯蔵物質の蓄積に関する理解は，人類の食糧確保或いは農業上極めて重要である．

種子は胚と胚乳，それらを包む種皮から構成されており，貯蔵物質の蓄積を担う胚乳の形成は極核が栄養受精することにより起こる．この胚乳の発達は種によって大きく異なり，イネやトウモロコシ，ヒマのような有胚乳種子となるものや，ダイズなどの無胚乳種子になるものがある．有胚乳種子では胚乳が貯蔵器官となり，これにデンプン，脂肪及びタンパク質をため込む．一方無胚乳種子では胚乳が初期に発達し後に消滅し，かわって子葉が貯蔵器官となる．

このような種子の貯蔵器官に蓄積される種子貯蔵タンパク質は人類をはじめ生物にとって貴重な窒素源であり，古くから研究されてきている．種子タンパク質はその性質から４つのグループ，水溶性アルブ

ミン，塩可溶性グロブリン，アルコール可溶性プロラミン及び酸またはアルカリ可溶性グルテリンに分類される (Osborne, 1924; Shewry, 1995)．またヒマのリシンに代表される糖タンパク質レクチンも種子内に蓄積している (Lord, 1985a)．これらの種子タンパク質の蓄積の鍵を握っているオルガネラとして登熟期の種子いわゆる貯蔵器官にプロテインボディが見られる．プロテインボディは単位膜で囲まれたオルガネラで，様々な種子タンパク質を蓄積している (Hara-Nishimura et al., 1982; Higgins 1984)．登熟期の種子細胞で生合成される種子タンパク質の輸送形態から，2種類に分類されている．

液胞タンパク質の細胞内輸送及びprotein-storage vacuole（プロテインボディ）の形成

種子タンパク質は合成量が多く，生化学的な解析に向いていることから，種子タンパク質をモデルにして，小胞体で合成された液胞タンパク質の液胞への輸送経路について多くの解析がなされてきた (Akazawa and Hara-Nishimura, 1985; Higgins, 1984)．そしてこれまでに，その種子タンパク質の細胞内輸送のルート及びその貯蔵場所であるプロテインボディが大きく分けて２通りあることが明らかにされている．１つ目はトウモロコシやイネのプロラミンで解析された．タンパク質を合成しているrERが徐々に大きくなっていき，最終的にそのままプロテインボディになる場合である(Burr and Burr, 1976; Larkins and Hurkman, 1978)．このルートは単子葉植物のみで知られており，これまで双子葉植物での報告はない．２つ目はrERで合成された種子タンパク質がデンスベシクルと呼ばれる小胞を経由して液胞まで運ばれる場合である．このルートはインゲンマメのファイトヘマグルチニン (Chrispeels, 1983)やカボチャの11Sグロブリン (Hara-Nishimura et al., 1985)と2Sアルブミン (Hara-Nishimura et al., 1993a) 及びヒマのリシン，

11Sグロブリン，2Sアルブミン (Lord, 1985a, 1985b) について知られている．そしてこれらのタンパク質が液胞に蓄積することによってプロテインボディが形成される (Johnson et al., 1989)．Chrispeelsのグループらは貯蔵器官に見られ，種子タンパク質を蓄積するプロテインボディの名をタンパク質貯蔵型の液胞 (protein-storage vacuole) とし，葉や根，茎といった栄養器官の液胞 (vegetative vacuole) と区別した (Johnson et al., 1989)．しかし最近オオムギやエンドウの根端細胞にもprotein-storage vacuoleとvegetative vacuoleが共存することが示された (Paris et al., 1996)．さらに登熟初期の種子細胞にも，この2つの液胞が共存することが判ってきた．

液胞の初期形成及び自食作用

液胞の発生については，その由来がゴルジ体であるという説も一部はあるが，一般的にはERから派生するとされてきている (Okita and Rogers, 1996)．エンドウ子葉の登熟初期の細胞の電顕観察から，ER由来と思われる管状の膜構造体の中に種子タンパク質が蓄積し始めている像や，この膜構造の発達によって生じたと考えられるprotein-storage vacuoleが，タンパク質を蓄積していない液胞を取り囲んでいる像が認められる (Robinson et al., 1995)．最近，その内側の液胞がvegetative vacuoleであることが，膜タンパク質の組成から明らかになった (Robinson et al., 1995)．この結果は単に，登熟初期の種子細胞中には2種類の液胞が存在することを示したというだけでなく，protein-storage vacuoleにはvegetative vacuoleを取り込んで分解する自食作用能が備わっていることを示唆した点で非常に興味深い．

動物ではリソソームが細胞内成分の分解の場として定義されている．細胞内成分を取り込んだ自食胞 (autophagosome) は加水分解酵素を含む後期エンドソーム (late endosome) と融合して成熟型リソソームとな

り細胞内成分の分解を行うことが広く知られている (Seglen and Bohley, 1992)．また，飢餓状態におかれた酵母の液胞が自食胞を取り込むことが報告され，この自食作用に関わる遺伝子が単離，解析されつつある(Takeshige et al., 1992; Tsukada and Ohsumi, 1993; Baba et al., 1994). これに対して，高等植物の液胞の自食作用については，殆ど知られていない．

しかし，植物細胞内でも細胞内成分を積極的に分解しなければならない時期がある．例えば種子が発芽する時であり，組織が退化する時であり，葉や花が老化する時である．このような時に細胞内不要産物の積極的な分解の鍵を握っているのが液胞であると考えられる．このような分解系に液胞が自食作用能を発揮するかどうかについて今後の解析により明らかになることが期待される．

植物液胞内で種子タンパク質の分解に関与するプロテアーゼ

種子の発芽時に起こる吸水に伴って，protein-storage vacuoleは互いに融合し，蓄積していた種子タンパク質を分解しつつ，大きなvegetative vacuoleへと変換していくことが示されている (Hara and Matsubara, 1980)．種子に蓄えられた種子タンパク質は，種子の発芽後，生長するための栄養源として使用され，植物体が十分に独立栄養が可能になるまでの必要不可欠の要素である．液胞内のいくつかのプロテアーゼがこの種子タンパク質の分解時に活性発現することが知られている．

種子タンパク質の分解は，基質特異性の高いエンドプロテアーゼによって初発の限定分解から始まる．そして，その結果できたペプチドが基質特異性の低いエンドペプチダーゼ及びエキソペプチダーゼによってアミノ酸レベルまで分解され，発芽生長のタンパク源として利用されると考えられている (Shutov and Vaintraub, 1987; Callis, 1995)．この中でも特に初発の限定分解を行うエンドペプチダーゼについて，プ

ロテインボディ (protein-storage vacuole) に局在することあるいはその活性増加と種子タンパク質の減少が関係するという2つの基準に該当するものについて調べられている．オオムギのプロラミンの分解に関わるシステインプロテアーゼ (Hammerton and Ho, 1986; Koehler and Ho, 1990) 及びダイズのグロブリンの分解に関わるセリンプロテアーゼ (Qi et al., 1992) は種子の発芽生長とともに活性が増加し，種子タンパク質の初発の限定分解を行うことが示されている．これに対してソバの種子タンパク質の分解に関わる金属プロテアーゼは登熟期の種子中にその基質である種子タンパク質とともにプロテインボディに存在する (Belozersky et al.,1990)．また，イネのシステインプロテアーゼインヒビターが種子の登熟期から発現してくることも報告されている(Kondo et al., 1990)．このことから，種子タンパク質分解に関わるプロテアーゼの活性発現の調節にインヒビターが関与していることも考えられる．このようなインヒビターをはじめとして，発芽生長に伴う種子タンパク質の分解は転写調節や翻訳後の様々な機構で調節を受けて起こっているにちがいない．しかしその機構については殆ど明らかになっていない．この一方で登熟期の種子中に存在する一部のプロテアーゼが種子タンパク質の生合成，その中でも翻訳後に起こるプロセシングに関与することが示されてきた．

種子タンパク質の成熟化と液胞プロセシング酵素

種子のprotein-storage vacuole内に蓄積しているアルブミンやグロブリン，リシンについては，そのcDNAの一次構造の解析とタンパク質のN末端アミノ酸配列の解析から，翻訳後にプロ型前駆体から成熟型に変換されることが明らかになってきた (Hara-Nishimura et al., 1993a, 1995)．前駆体タンパク質が受けるプロセシングについて，ヒマのリシン (Harley and Load, 1985)，カボチャの11Sグロブリン (Hara-Nishimura et

al., 1987), ヒマの11Sグロブリン (Hara-Nishimura et al., 1987), ダイズのグリシニン (Scott et al., 1992; Muramatsu and Fukasawa, 1993) などの前駆体をプロセスする酵素が別々に解析されてきた．これに対して，ヒマから精製された11Sグロブリン前駆体のプロセシングを行う酵素が，11Sグロブリン以外の他の種子タンパク質を行うことが示された (Hara-Nishimura et al., 1991)．このことから複数の種子タンパク質の成熟化を一種類の酵素が行うことが明らかとなり，この酵素が液胞プロセシング酵素 (vacuolar processing enzyme: VPE) と名付けられた．ヒマVPEのcDNAクローンが単離され，それを用いた解析が行われた結果，VPEはパパイン(Cohen et al., 1986)をはじめとするパパインファミリーに属さないことがわかった．唯一ヒトに寄生する住血吸虫の推定プロテアーゼSm32 (Klinkert et al., 1989)と相同性が見つかる，新規のシステインプロテアーゼであることが明らかにされた (Hara-Nishimura et al., 1993b)．既知のプロテアーゼのほとんどが不活性型前駆体として合成され，なんらかの機構によって活性発現することが示されてきている(Neurath, 1989)．これと同様にヒマVPEのcDNAを用いた解析 (Hara-Nishimura et al., 1993b) 及びSm32をSf9で発現させた解析 (Gotz and Klinkert, 1993) から，VPEの不活性型前駆体及びその活性発現機構の存在が示唆されている．

またナタマメからの精製酵素を用いた解析より，VPEがアスパラギン特異的なエンドペプチダーゼであることが示された (Abe et al., 1993)．種子タンパク質前駆体のプロセシングの部位が，アスパラギン残基のC末端側であるものがほとんどである．そしてそのプロセシングの部位は親水性の領域に局在している．このことから，種子タンパク質前駆体の表面に露出しているアスパラギン残基をVPEが切断しているという種子タンパク質のプロセシング機構が提唱された (Hara-Nishimura et al., 1993a)．つまりVPEがアスパラギン特異的なエンドペプチダーゼで，接近できうるアスパラギン残基があればそのペプチド

結合を切断するという考え方である．この考え方は立体構造の明らかになっているナタマメのコンカナバリンAなどの表面にはアスパラギン残基はほとんどないことや尿素で立体構造をほぐした種子タンパク質は精製VPEにより小さなペプチドにまで分解されるということから支持される．このように殆どの種子タンパク質の成熟化に，VPEによるプロセシングが働くものと考えられる．また，これと同様のプロセシングの系が本葉や根といった栄養器官の液胞タンパク質の生合成過程にも起こることが示唆されている．

液胞タンパク質の成熟化機構へのアスパラギン酸プロテアーゼ(AP)の関与

種子タンパク質の中で2Sアルブミンはその生合成について最も解析が進められているタンパク質のうちの１つである．これまでに報告されている2Sアルブミン間で保存されている２つのアスパラギン残基のC末端側でVPEによるプロセシングを受け，成熟型になると考えられている(Hara-Nishimura et al., 1993a)．しかし，ブラジルナッツの2Sアルブミンは，２つのうち一方のアスパラギン残基を欠き，成熟型タンパク質になる前に長い期間中間体のままで存在することが知られている(Sun et al., 1987)．また，シロイヌナズナ (Krebbers et al., 1988)，ナタネ (Ericson et. al., 1986; Monsalve et al., 1991), ヒマ (Irwin et al., 1990; Sharief et al., 1982)，ブラジルナッツ (Ampe et al., 1986; Altenbach et al., 1992) の2Sアルブミン前駆体は成熟型タンパク質の２つのサブユニットの間に成熟型には見られないプロペプチドを持つ．このペプチドは恐らくプロテアーゼによって分解されると考えられる．種子タンパク質の生合成の過程でVPEによるプロセシング後に残るプロ領域の除去が必要となってくると思われるが，詳細な解析はなされていない．

酵母ではプロテイナーゼAがカルボキシペプチダーゼYをはじめとす

る液胞タンパク質の成熟化に関与すると考えられている(Ammerer et al., 1986; Jones et al., 1982; Woolford et al., 1986). このプロテイナーゼAや動物のカテプシンDと同様のプロテアーゼであるアスパラギン酸プロテアーゼが植物種子にも存在することが報告されている. (Bond and Butler, 1987; Sarkkinen et al., 1992). またRuneberg-Roos et al.(1994), Paris et al. (1996)らは免疫電顕観察などにより, オオムギのアスパラギン酸プロテアーゼが本葉や根の細胞の液胞 protein-storage vacuoleに局在することを示している. このことからAPが液胞タンパク質の成熟化に関与することが十分考えられる.

そこで今回の研究では液胞タンパク質の成熟化機構について明らかにするため, ヒマ種子からAPの精製を行い, その性質についての解析や精製酵素を用いたプロセシング実験を行った. またこれと平行してヒマVPEについてヒマ種子細胞内での局在性及び植物液胞一般でのVPE活性の存在について検討した. そしてこれらの結果を基にヒマ種子液胞内での液胞タンパク質の成熟化機構におけるVPE及びAPの役割について考察した. また後半部では酵母細胞でVPEの発現解析を行い, VPEの活性発現機構について考察した. このような解析により, 植物液胞内におけるVPEやAPの機能について, さらにそれらが存在する植物液胞の性質及びその機能について新たなる知見が得られれば幸いである.

材料及び方法

Protein-storage vacuole（プロテインボディ）の単離及び分画

乾燥ヒマ種子より，Hara-Nishimura et al.(1992)の方法に従いグリセロール法によってprotein-storage vacuoleを単離した．殻を除いた乾燥ヒマ種子5 gに8 mlのグリセロールを加えブレンダーで2 分間ホモゲナイズした後，90,000 x g（Beckman, JA-20, 15,000 rpm ），15 ℃で15 分間遠心した．Protein-storage vacuoleは非常に密度の高いオルガネラであるため，グリセロール中で遠心することにより，他のオルガネラより分画される．沈殿として得られたprotein-storage vacuoleはグリセロールへの再懸濁と遠心を2回繰り返すことによって精製し，最終標品とした．得られたprotein-storage vacuoleは光学顕微鏡による観察とmarker enzymeの活性測定により，intactで他のオルガネラや細胞質の構成要素の混成はごく少量であることが示されている．これに10 mM Tris-HCl (pH 7.5)を1 ml加え懸濁した後，100,000 x g，4 ℃で20分間遠心し，得られた上清をマトリクス画分，沈殿を主要種子タンパク質である11Sグロブリンから構成されクリスタロイド画分とした．

AP及びVPEの精製

APの精製はSarkkinen et al. (1992) の方法に従い，ペプスタチンA-アガロースを用いたアフィニティークロマトグラフィー法で行った．前述の方法で得られたprotein-storage vacuoleのマトリクス画分に50 mM sodium acetate (pH 3.5)， 0.5 M NaClのバッファーを加え，遠心して不溶性画分を除いたあとにペプスタチンA-アガロースカラムにかけた．カラムを50 mM Tris-HCl (pH 7.5)，0.5 M NaCl のバッファーで洗ったあと50 mM sodium bicarbonate (pH 10)，0.5 M NaCl のバッファーで溶出した．VPEの精製はHara-Nishimura et al. (1991)の方法に従って，同じくprotein-storage vacuoleのマトリクス画分を出発材料として行った．

APのタンパク質のN末端アミノ酸配列の決定

精製酵素はSDS-PAGE（15%ゲル）泳動後ゲル中のタンパク質をProBlott膜 (Applied Biosystems, USA)に電気的に転写した．APの各サブユニットに相当するバンドを切り出し，peptide sequencer (model 476A, Applied Biosystems)でEdman分解に供し，アミノ酸配列を決定した．

AP及びVPEに対する特異抗血清の調製

AP及びVPEの精製は前述した通りに行った．各精製酵素を等量のcomplete Freund's adjuvantとよく混合し乳濁化した．これを第1回目としてウサギに免疫感作した．1回目の免疫感作から約3週間後にincomplete Freund's adjuvantを用いて2回目の免疫感作，さらにその1週間後に3回目の免疫感作を行い，その1週間後に採血した．一方ではAP及びVPEとmaltose-binding proteinとの融合タンパク質を大腸菌で発現させ，できたタンパク質をSDS-PAGEにかけた．泳動後のゲルをCoomasie Blue R-250で染色し，相当するバンドを切り出して，同様の方法で抗体作製を行った．抗カルボキシペプチダーゼ (CPY) 抗血清は東大の和田先生から頂いた．

SDS-PAGE及びイムノブロット解析

SDS-PAGEはLaemmli (1970) の方法に準じて行った．タンパク抽出液は試料溶解液 (2% SDS, 50 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 0.1% BPB, 10% glycerol) に懸濁し，95 ℃で5分間加熱処理し，泳動に用いた．泳動後，ゲル中のタンパク質をBurnette (1981) の方法に準じてGVHP膜 (Millipore) に電気的に転写した．転写にはセミドライタイプの装置 (ATTO) を用いて，2 mA/cm^{2}で1時間行った．抗体の非特異的吸着を防ぐため，この膜を5% (w/v) スキムミルクを含むTBS-Tween溶液 [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (w/v) Tween 20] 中で1時間ブロッキング処理し，膜をTBS-Tween溶液で15分間1回，5分間

2回洗浄した後にそれぞれの抗血清を1000倍から5000倍希釈になるように加えて1時間振とうした．その後，膜をTBS-Tween溶液で15分間1回，5分間2回洗浄し，5000倍希釈のセイヨウワサビペルオキシダーゼ結合抗ウサギIgG抗体 (Amersham) を含むTBS-Tween溶液中で30分間振とうした．膜をTBS-Tween溶液で15分間1回，5分間2回洗浄した後，1分間発色溶液中で振とうさせた．発色反応させた膜を10秒から2分間X線フィルムと接触させた後にX線フィルムを現像することにより各抗血清と反応性のあるタンパク質を検出した．

*In vitro*でのER内のプロ型タンパク質のプロセシング

Hara-Nishimura et al. (1991)の方法に従って，登熟ヒマ胚乳にTRAN^{35}S-Label (胚乳１つあたり1.9 MBq；ICN Biomedicals Inc.)を塗って1時間パルスラベルした．その後コールドの10 mMメチオニン及びシステイン溶液で3度洗浄し，水で湿らせた濾紙の上に1時間おいてチェイスした．その胚乳を抽出緩衝溶液 [150 mM Tricine-KOH, pH 6.5，1 mM EDTA，13% (w/w)ショ糖]を加えながら氷上でカミソリを用いて細かく破砕し，その破砕液を1層のガーゼで濾過したあと，不連続なショ糖密度勾配液に重層し，スイングローターを用いて4 ℃，60,000 x g で1時間遠心した．ER画分と液胞画分を回収して，以下の*in vitro*でのプロセシング実験に用いた．^{35}Sでラベルされた種子タンパク質前駆体が含まれているER画分にVPEまたはAP (各0.4 mU)，反応緩衝溶液 (0.1 M sodium acetate, pH5.5，0.1 M dithithreitol，0.1 mM EDTA)を加えて37 ℃で5分から30分反応を行った．反応溶液を15%アクリルアミドゲルを用いてSDS-PAGEに供したあと，Bio-Imaging Analyzer (BAS 2000; Fuji Film) で解析した．各前駆体及び成熟型タンパク質は免疫沈降及びその分子量から同定した．

APによる合成ペプチドの切断部位の決定

シロイヌナズナ2Sアルブミン-2前駆体のプロセシングサイトを含むプロペプチド部分の配列をもとに18アミノ酸残基からなる合成ペプチドLE-18 (PSLDDEFDLEDDIENPQG; Krebbers et al., 1988)をペプチド合成機 (model 430A, Perkin Elmer/Applied Biosystems)で合成した．100 nmoleの合成ペプチドに精製酵素及び反応緩衝溶液 (0.1 M citrate phosphate buffer, pH 3.0) を加え，37 ℃で30分間反応を行った．反応によって生じたペプチド断片をHPLCで分離し，得られた各ピークの画分のアミノ酸組成をHitachi 835 Amino Acid Analyzerで決定した．

APのプロセシング活性の検出

基質である6 nmoleのLE-18にAPを含む抽出液及び反応緩衝溶液(20 mM sodium acetate, pH3.0，0.1 mM EDTA)を加えて37℃で10分から30分間反応を行った．反応溶液はキャピラリー電気泳動(model 270A；Applied Biosystems) へ30 ℃，20kV， 10 mM sodium borate buffer (pH 9.0) の条件でかけ，200 nmの吸光度で測定した．APはLE-18を複数のサイトで切断するため，反応後P1からP5までの５本のピークが検出される．このうちP2を指標として，基質1マイクロモルを1分間に切断する酵素活性を1ユニットとした．

cDNAクローンの単離と塩基配列の決定

cDNAライブラリーはMori et al. (1991)の方法に従って，登熟カボチャ子葉より調整したpoly(A)$^+$RNA及びベクターpTTQ18(Amersham)を用いて作製した．これまでに報告されているAPの活性中心と予想される保存されたアミノ酸配列をもとにオリゴヌクレオチド

5'-GATACTGGTTC(GATC)TC(GATC)AA(CT)CTITGGGTICC-3'をデザインし，DNA合成機 (Pharmacia LKB Biotechnology) で化学合成した．

合成オリゴヌクレオチドをプローブとしてコロニーハイブリダイゼーションにより，登熟カボチャ子葉cDNAライブラリーをスクリーニングした．得られたポジティブクローンを用いて更にスクリーニングを繰り返して全長を含むクローンを単離した．cDNAインサートを制限酵素で断片化してベクターにサブクローンし，これを塩基配列決定に供した．塩基配列はDye Primer Cycle Sequencing Kit (Perkin Elmer/Applied Biosystems)によって反応を行い，DNAシークエンサー(model377；Applied Biosystems)を用いて解析した．

電子顕微鏡観察

試料の作製はNishimura et al. (1993) の方法に従って行った．登熟ヒマ胚乳組織を固定液 (4% paraformaldehyde, 1% glutaraldehyde, 0.05 M cacodylate buffer, pH7.4, 0.06 M sucrose)で 1 時間減圧浸透処理した後に 1 mm角以下に細切りし，新しい固定液でさらに2時間処理し，ジメチルホルムアミド濃度勾配系列により-20 ℃で脱水した．その後，試料はLR-White樹脂（London Resin）に包埋し，紫外線重合装置（堂阪イーエム）を用いて重合させた．そして，ミクロトームで切片を作製し，ニッケルグリッドにマウントした．切片をブロッキング液（1% BSA in PBS）で室温で1時間処理した後，ブロッキング液で500倍希釈した各抗血清と4 ℃で一晩反応させた．PBSで洗浄後，切片はブロッキング液で20倍希釈したプロテインA一金コロイド液（Amersham）で室温で30分処理した．切片は蒸留水で洗浄し，4% 酢酸ウランとクエン酸鉛で染色した．染色後透過型電子顕微鏡（JEOL 1200EX）を使用して 80 kVで観察した．

各植物組織及び器官からのタンパク質の抽出

実験材料としてヒマ，ヤエナリ，カボチャ，コメ，ダイズ及びホウレンソウを用いた．登熟ヒマ種子胚乳は1992年及び1993年にかけて温

室で生育させた植物体より得た．登熟初期（ステージI）は開花後15日から20日，登熟中期（ステージII）は開花後25日から30日，登熟後期（ステージIII）は開花後35日から40日の種子を示す．カボチャ，イネ，ダイズ登熟種子及びホウレンソウ本葉は農場で生育させた植物体より得た．発芽ヒマ，カボチャ種子の胚乳，根及び胚軸は乾燥種子を流水中で一晩吸水させた後湿らせたバーミキュライト中に播き，25 ℃暗下で生育させた植物体より得た．ヤエナリ種子は水の上の網で25 ℃暗下で生育させた植物体から根及び胚軸を得た．

胚乳及び子葉は1 gあたり2 mlの10 mM Tris-HCl（pH 7.5）を加えて氷上の乳鉢ですりつぶした．胚軸，根, 本葉は1 gあたり0.5～2 mlの0.1 M dithiothreitol，1 mM EDTAを含む0.1 M sodium acetate（pH 5.5）を加えて氷上の乳鉢ですりつぶした．各液を4 ℃ 20,000 x gで15分間遠心し，遠心上清をさらに同様の条件で遠心して，得られた上清をタンパク質抽出液として以降の実験に供した．タンパク質量はProtein Assay Kit (Bio-Rad,USA)を用いて測定した．

VPE活性の測定法

VPE活性の測定はHara-Nishimura et al. (1991) の方法に従って行った．カボチャの主要種子タンパク質である11Sグロブリンのプロ型前駆体タンパク質であるプログロブリンのプロセシングサイトを含むアミノ酸10残基（Ser-Glu-Ser-Glu-Asn-Gly-Leu-Glu-Glu-Thr）からなる合成ペプチド(NG-10)を基質として用いた．20 mM sodium acetate (pH 5.5), 0.1 M dithiothreitol，0.1 mM EDTAからなるバッファー中に4.5 nmolの基質と抽出液を加え，37 ℃で10 分から30 分反応させ，キャピラリー電気泳動(model 270A； Applied Biosystems) 30 ℃，20kV， 10 mM sodium borate buffer (pH 9.0) の条件でかけ，200 nmの吸光度で測定した．VPEは基質である合成ペプチド(NG-10)のアスパラギン残基のC末端側のペプチド結合のみを切断する．その結果，NG-10にかわってN

末端側のpentapeptide P1とC末端側のpentapeptide P2が生じる．このP2を指標として，基質1マイクロモルを1分間に切断する酵素活性を1ユニットとした．

発現コンストラクトの構築と酵母細胞の形質転換

酵母（*Saccharomyces cerevisiae*)のYW23-5A (MATa leu2 ura3-52)とYW7-6D (MATa leu2 ura3-52 pep4-3)ウラシル要求性各株を用いた．酵母での発現ベクターとして，酵母及び大腸菌のシャトルベクターpYES2 (Invitrogen)を用いた．

登熟ヒマ胚乳ライブラリーより単離したVPEのcDNAの塩基配列を基に，開始コドン周辺の配列及びPvuIIの認識配列を含むオリゴヌクレオチド，或いは終止コドン周辺の配列及びXhoIの認識配列を含むオリゴヌクレオチドをデザインしてDNA合成機 (Pharmacia LKB Biotechnology)で化学合成した．合成オリゴヌクレオチドをプライマーとして組み合わせ，VPEのcDNAを鋳型としてPfu DNAポリメラーゼ（Stratagene）とともにPCRに供した．その後，増幅したDNA断片を回収し，PvuII及びXhoIで消化してベクターに連結し，大腸菌を形質転換した．形質転換体のプラスミドを増幅して回収し塩基配列を確認後，酵母細胞の形質転換に用いた．

プラスミドをウラシル要求性酵母株にIto et al. (1983)，Schiestel and Gietz (1989)の方法に従って形質転換した後，0.002%アデニン塩，0.002%L-トリプトファン，0.002%L-ヒスチジン塩，0.003%L-ロイシン，0.003%L-リジンを含むSD寒天プレート(0.17% bacto-yeast nitrogen base without amino acids, 2% glucose, 2% bacto-agar)に播いて30 ℃で2日間培養し，ウラシル非要求性株を選抜した．コロニーをWada et al. (1992)の方法に従い，0.002%アデニン塩，0.002%L-トリプトファン，0.002%L-ヒスチジン塩，0.003%L-ロイシン，0.003%L-リジンを含むラフィノース液体培地(0.17% bacto-yeast nitrogen base without amino acids,

2%ラフィノース，0.5%カザミノ酸）に懸濁し，細胞が定常状態に達するまで30 ℃で培養した後細胞を回収し，上述の液体培地のラフィノースをガラクトース（5%）或いはグルコース（2%）に置換した培地に移して2時間或いは30時間培養した．

細胞抽出液の調製

ガラクトース或いはグルコース存在下で生育させた細胞(約$1X10^8$)を回収し，10 mM PMSFを含むSDS変性緩衝液中または20 mMTris-HCl (pH 7.5)中でグラスビーズ (Sigma) を用いて磨砕した後遠心し，抽出液をSDS-PAGEまたは活性測定に供した．

細胞分画

酵母の細胞分画はNishikawa and Nakano (1991)の方法，Horazdovsky and Emr (1993)の方法に従って行った．

変異VPEの作製

変異VPEの作製は，site-directed mutagenesis system, Mutan-Express^R Km (TaKaRa)を用いて行った．プラスミドベクターpKF19kはカナマイシン耐性遺伝子上に2重のアンバー変異を持っているため，宿主菌として*supE*株を利用した場合にのみ増幅が可能となる．このベクターにヒマVPEをクローニングし，熱処理によって一本鎖を形成させた後，目的の変異をいれて合成したオリゴヌクレオチドとカナマイシン耐性遺伝子上のアンバーを復帰させるためのセレクションプライマーを同時にハイブリダイズさせる．続いて，ポリメラーゼ/リガーゼ反応によって，相補鎖を合成し*supE/mut*S株に導入すると，DNAはミスマッチ修復を抑えながら複製を行う．さらにここから，*sup*^{0}株でのみ増幅できるDNAを選択することにより，目的の変異が導入されたDNAを得ることができる．

塩基配列の決定はヒマVPEのcDNA塩基配列に基づいてオリゴヌクレオチドを合成し，これを使ってDye Terminator Cycle Sequencing Kit (Perkin Elmer/Applied Biosystems)によって反応を行い，DNAシークエンサー(model377；Applied Biosystems)を用いて解析した.

結　果

アスパラギン酸プロテアーゼ(AP)のヒマ種子protein-storage vacuoleの可溶性マトリクスからの精製

APの特異的阻害剤であるペプスタチンAは酸性条件下で活性型のAPと強く結合し，その結合はpHを上げることによって解離する(Umezawa, 1976)．これを利用して，ペプスタチンA-アガロースを用いたアフィニィティークロマトグラフィーにより，ヒマ種子胚乳から単離したprotein-storage vacuoleのマトリクス画分を出発材料として本酵素の精製を行った．

最終精製タンパク質をSDS-PAGEにかけCBB染色を行ったところ，48 kDaと29 kDaの2つのバンドが検出された（図1 a，lane 2）．このタンパク質に還元剤を加えると48-kDaタンパクが32 kDaと16 kDaのサブユニットに分離した（図1 a，lane 1）．このことは48-kDaタンパクが1つかまたはそれ以上のジスルフィド結合によって2つのサブユニットから構成されることを示している．これに対して29-kDaタンパクは還元剤を加えても変化はなかった．29-kDaサブユニットがAPのアイソフォームであるのか，32-kDaサブユニットの分解産物であるのか調べるため，32-kDa及び29-kDaサブユニットのN末端アミノ酸配列を決定した．その結果，29-kDaサブユニットのN末端アミノ酸配列(GDSKDTDIVALKNYLDAQY) は32-kDaサブユニットのアミノ酸配列(DAFDTDIVALKNYLDAQY)とは異なっていた（図1 b）．32-kDaサブユニットのN末端のアミノ酸は29-kDaサブユニットの2番目のアミノ酸に相当する．また，32-kDaサブユニットのN末端側から2番目のアラニン，3番目のフェニルアラニンは29-kDaサブユニットでは3番目のセリン，4番目のリジンに変わっていた．この結果からヒマ種子胚乳には2つのAPのアイソフォームが存在すると考えられた．さらにこのことは29-kDaサブユニットがジスルフィド結合に必要なシステイン残基を欠

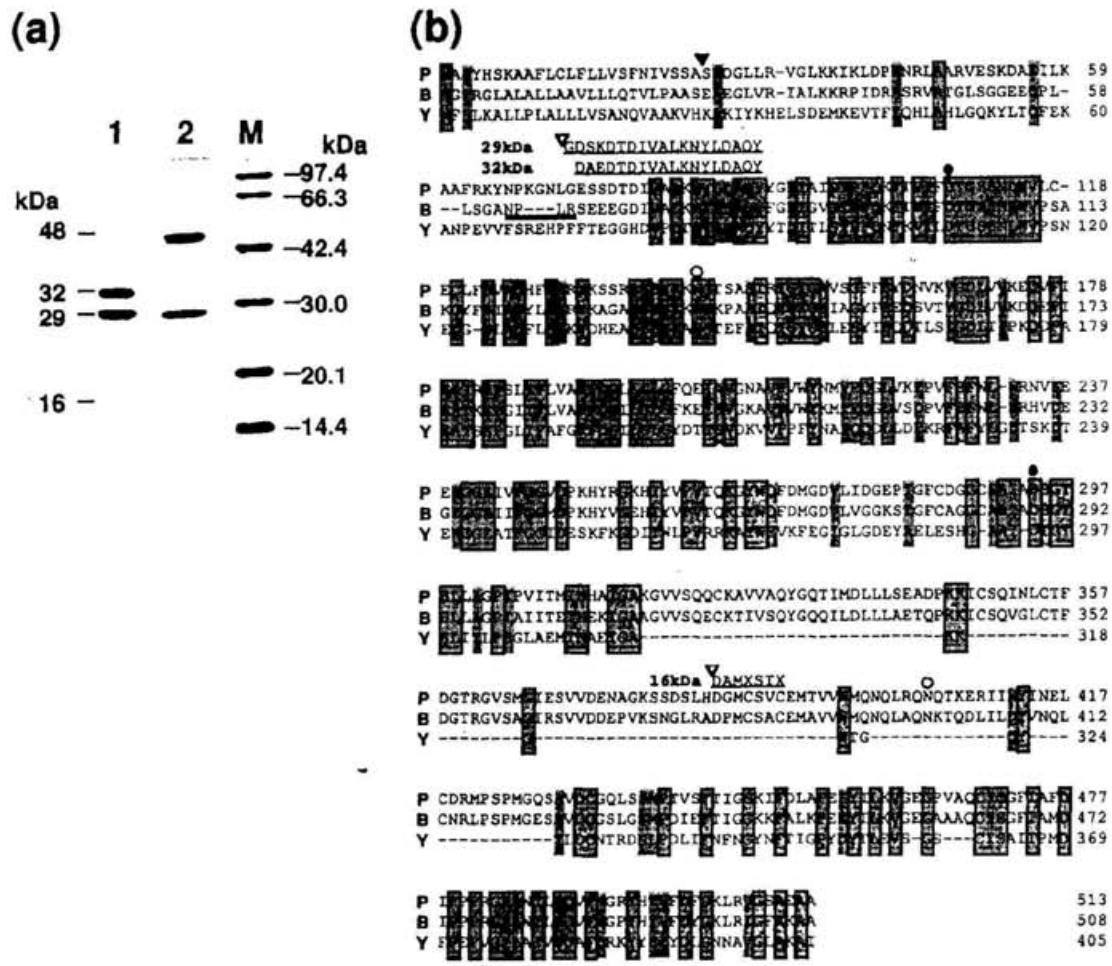


図１．ヒマ種子胚乳から精製したAP

(a) ヒマprotein-storage vacuoleから精製した酵素を還元剤を入れた状態 (lane 1) 及び還元剤を入れない状態 (lane 2)でSDS-PAGE後，CBB染色を行った．Mは分子量マーカーを示し，右側の数字はマーカータンパク質の相対質量 (kDa) を示す．(b) ヒマ酵素の29 kDa及び32 kDaタンパク質のN末端アミノ酸配列と他の植物，カボチャ（P，双子葉），オオムギ（B，単子葉）及び酵母（Y）のAPの配列と比較した．液胞へのターゲッティングシグナルであるNPLR配列はオオムギ酵素の二重下線で示したところに見られるが，カボチャ酵素からは見られない．黒三角はシグナルペプチドの切断が起こると予想される位置，白三角は翻訳後の切断が起こると予想される位置，黒丸はAPの活性中心，白丸はN結合型糖鎖の付加が起こると予想される位置を示す．相同なアミノ酸を四角で囲み，類似のアミノ酸を網掛けにした．

いていることを示唆している．APの構造を決定するために双子葉のカボチャの登熟子葉からAPのcDNAクローニングを行った．得られたcDNAクローンは全長1776bp，513アミノ酸残基のタンパク質をコードしうるオープンリーディングフレームを含んでいた（図1b）．このカボチャAPのアミノ酸配列のホモロジー解析を行った結果，カボチャ酵素は単子葉であるオオムギのAP (Runeberg-Roos et al., 1991)と66%，酵母酵素(Ammerer et al., 1986)と31%の同一性が見られた．今回精製したヒマAPの各サブユニットのN末端アミノ酸配列は，オオムギ酵素よりカボチャ酵素の配列の方に類似していた．また16-kDaサブユニットのN末端はそのN末端アミノ酸配列 (DAXSTX, Xは検出できなかったアミノ酸)から，カボチャ酵素前駆体の開始メチオニンから384番目のアスパラギン酸残基に相当すると思われる（図1b）．カボチャ酵素前駆体の一次構造の特徴としてN末端にシグナルペプチドと考えられる疎水領域が見られた．von Heijne (1986) の報告に従うとカボチャ酵素前駆体は25番目のアラニンのC末端側で翻訳と同時にシグナルペプチドの切断を受けると考えられる．さらにヒマ精製酵素のN末端アミノ酸配列との比較からカボチャ酵素はN末端側及び内部にプロ領域をもつ前駆体として合成されると考えられる．つまり翻訳後にN末端側及び内部のプロ領域の切断を受けて，32 kDaと16 kDaのサブユニットができることが予想される．これに対して29-kDaサブユニットは32-kDaサブユニットのN末端アミノ酸残基の1つ前のところで切断されて生じると考えられた（図1b）．オオムギ酵素は切断部位の少し前にNPLR配列を持ち，その配列が液胞への輸送シグナルであると考えられている（図1b，Runeberg-Roos et al., 1994）．しかしカボチャ酵素ではその配列は見られなかった．

APのprotein-storage vacuole内の局在性及び細胞内局在性

乾燥種子に見られるprotein-storage vacuoleは単位膜で囲まれたオルガ

ネラで，種子の登熟に伴い液胞が変換してできてくることが示されている (Hara-Nishimura et al., 1987)．ヒマ種子のprotein-storage vacuoleは封入体として主要種子タンパク質である11Sグロブリンの結晶構造体であるクリスタロイドをもつ (Tully and Beevers, 1976; Hara-Nishimura et al., 1982)．植物種子のAPのprotein-storage vacuole内での局在性について調べるために，ヒマ種子からの精製酵素を抗原としてこれに対する抗体を調製した．Protein-storage vacuoleを乾燥ヒマ種子から単離しSDS-PAGEにかけこの抗体を用いてイムノブロット解析を行ったところ，還元剤のない状態で48 kDaのバンドが検出された（図２，lane 4）．この48 kDaのバンドは精製酵素の分子量と一致したことから，この抗体はAPに特異的に反応していると考えている．この液胞は11Sグロブリンの結晶構造体であるクリスタロイドを含んでおり，得られた液胞をバーストさせ遠心することによって可溶性マトリクス画分と不溶性クリスタロイド画分（膜画分を含む）とに分画することができる．得られた２つの画分をSDS-PAGEにかけ，APに対するイムノブロット解析を行ったところ，APを示す48 kDaのバンドは可溶性マトリクス画分からのみ検出された（図２，lane 5）．

さらに本酵素の細胞内局在性について調べるため，AP及び11Sグロブリンに対する抗体を用いて登熟後期のヒマ種子の免疫電顕観察を行った．11Sグロブリンを示す金粒子は可溶性マトリクス領域及び不溶性クリスタロイド領域ともに局在している（図３b）．これに対してAPを示す金粒子はprotein-storage vacuoleの不溶性クリスタロイド領域ではなく可溶性マトリクス領域に局在しており，イムノブロット解析による結果と一致した（図３a）．以上の結果からAPは種子の登熟期には主要種子タンパク質とともにprotein-storage vacuoleに局在することが明らかになった．

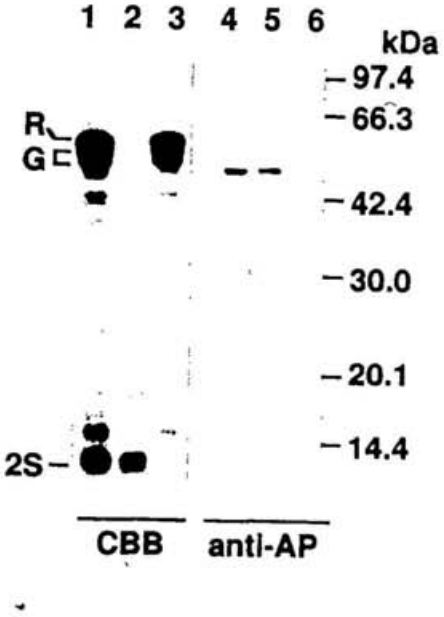


図２．ヒマ種子胚乳protein-storage vacuole内の可溶性マトリクス画分中のAPの局在

ヒマ種子胚乳から単離したprotein-storage vacuole (lanes 1, 4) 及びその可溶性マトリクス画分 (lanes 2, 5)，不溶性クリスタロイド画分 (lanes 3, 6) を還元剤を入れない状態でSDS-PAGE後，CBB染色 (lanes 1-3)または抗AP抗体によるイムノブロット解析 (lanes 1-3) を行った．RはRCAとリシン，Gは11Sグロブリン， 2Sは2Sアルブミンを示す．右側の数字はマーカータンパク質の相対質量 (kDa) を示す．

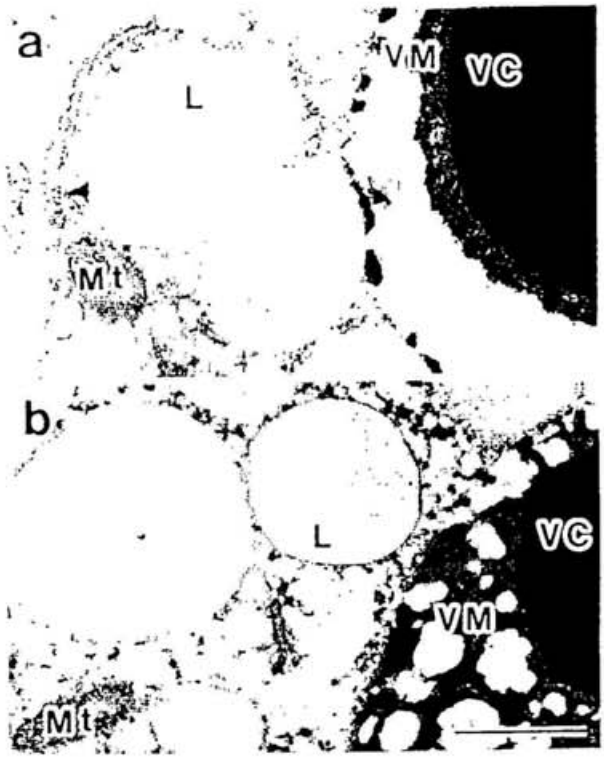


図３．登熟ヒマ胚乳のprotein-storage vacuole内のAPの局在
抗AP抗体 (a) 及び抗11Sグロブリン抗体 (b) による登熟ヒマ種子胚乳の免疫電顕観察．デンスベシクルは arrowheadsで示す．VM, VC, L, Mtはそれぞれ液胞可溶性マトリクス，液胞不溶性クリスタロイド，リピッドボディ，ミトコンドリアを示す．バーは１μmである．

種子の登熟期から発芽生長期にかけてのAPのタンパク量の変動

種子の登熟期には種子タンパク質の生合成及び蓄積がさかんに起こる．この時期の液胞内に他の種子タンパク質とともにAPが局在することは，本酵素が種子タンパク質の成熟化に関与する可能性が考えられる．そこでヒマ種子胚乳の登熟期から発芽生長期におけるAPのタンパクレベルの変動について調べた（図４）．ヒマ種子の種子タンパク質は開花後30-40日後ステージIIIに最も盛んに合成され蓄積する（図４a）．APは，この種子タンパク質の蓄積より先のステージIIから増加した(図４b, c）．また，発芽生長期にはその酵素量は減少し，発芽２日以降に起こる種子タンパク質の急速な分解と一致していた（図４b, c）．この結果はAPが種子タンパク質の分解というよりもむしろ成熟化に関与して，役割を果たしていることを示唆している．

VPEのprotein-storage vacuole内での局在性

ヒマ種子protein-storage vacuoleから精製されたVPEが種子タンパク質前駆体のプロセシングを行うことが示されている (Hara-Nishimura et al., 1982)．植物種子のprotein-storage vacuole内でのVPEの局在性について調べるため，本酵素に対する特異抗体を調製した．APの時と同様に乾燥ヒマ種子からprotein-storage vacuoleを単離し，可溶性のマトリクス画分と不溶性のクリスタロイド画分へと分画し，本酵素に対する特異抗体を用いたイムノブロット解析を行った．VPEはAPと同様protein-storage vacuoleのマトリクスに局在することが判明した（図５）．マトリクス画分から検出されたバンドは37 kDa，41 kDaで，37-kDaタンパク質は精製酵素の分子量と一致した (Hara-Nishimura et al., 1991)．この41 kDaタンパク質はイムノブロット解析に用いる抗体を大腸菌で発現させたmaltose-binding proteinとVPEとの融合タンパク質に対する抗体に変えても同様の結果が得られた．このことから41 kDaのタンパク質も本酵素に関係していることが示唆された．しかし，この41 kDa

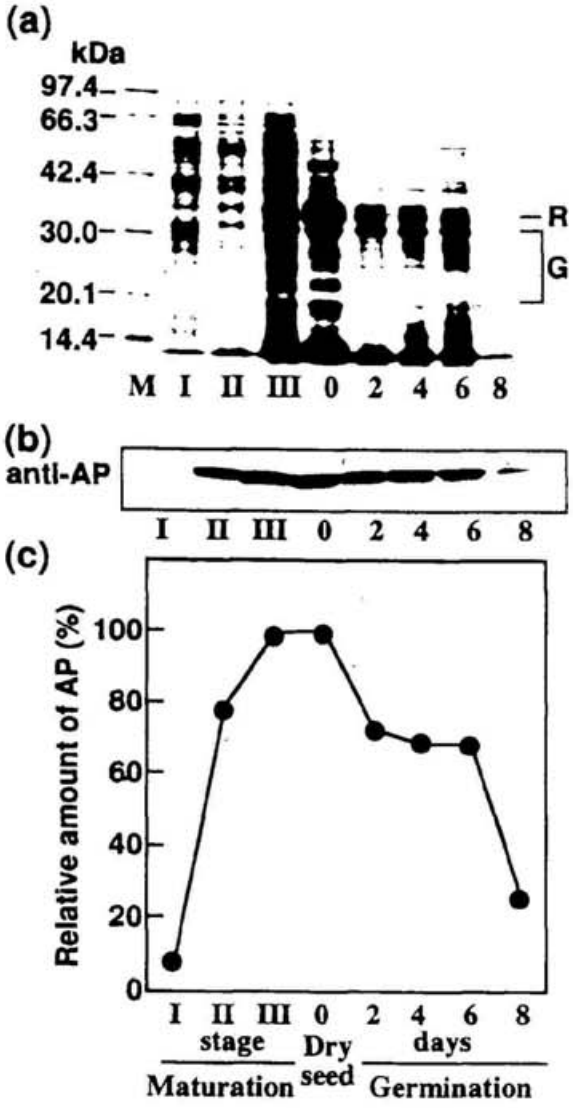


図4．ヒマ種子の登熟期 (Maturation) から乾燥期 (Dry seed) 及び発芽生長期 (Germination) にかけてのAPのタンパク量の変動

(a) 各ステージのヒマ種子胚乳からのタンパク抽出液を還元剤を入れた状態でSDS-PAGE後，CBB染色を行った．登熟ステージI，II，III はそれぞれ登熟前期，中期，後期，0は乾燥種子，発芽生長期2，4，6，8はそれぞれ播種後の日数を示す．RはRCAとリシン，Gは11Sグロブリンを示す．Mは分子量マーカーを示し，左側の数字はマーカータンパク質の相対質量 (kDa) を示す．(b) 各ステージのヒマ種子胚乳からのタンパク抽出液を還元剤を入れない状態でSDS-PAGE後，抗AP抗体を用いてイムノブロット解析を行った．(c) (b) の結果をデンシトメーターで測定し酵素タンパク質の最大量に対する割合を数値化した．

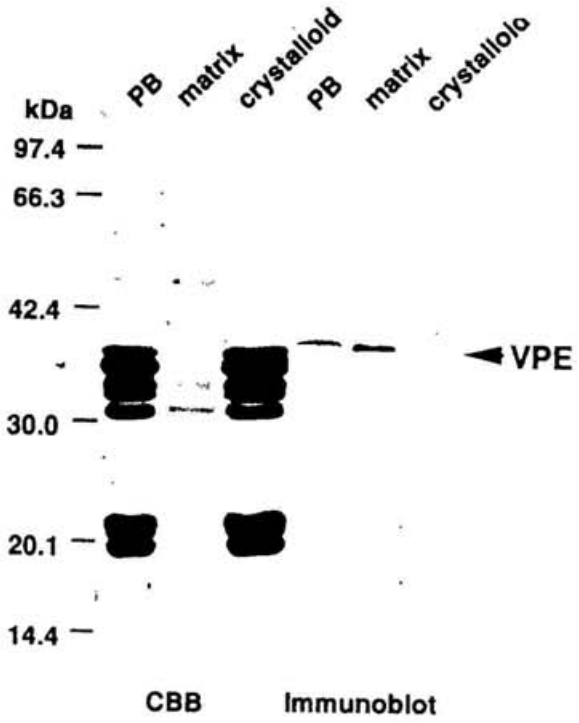


図５．ヒマ種子胚乳protein-storage vacuole内の可溶性マトリクス画分中のVPEの局在

ヒマ種子胚乳から単離したprotein-storage vacuole (PB) 及びその可溶性マトリクス画分 (matrix)，不溶性クリスタロイド画分 (crystalloid) をSDS-PAGE後，CBB染色（左側パネル）または抗VPE抗体を用いたイムノブロット解析（右側パネル）を行った．左側の数字はマーカータンパク質の相対質量 (kDa) を示す．

のタンパク質が実際に活性を持つかどうかは不明である．登熟ヒマ種子胚乳の免疫電顕観察 (Hara-Nishimura et al., 1993b)や登熟カボチャ子葉から単離したprotein-storage vacuoleのサブオルガネラレベルでの分画からVPEはprotein-storage vacuoleのマトリクスに局在することが示されている(Hara-Nishimura et al., 1993b, Hara-Nishimura and Nishimura, 1987)．ヒマprotein-storage vacuoleのマトリクスにVPEが局在することが示され，VPEが種子細胞の液胞で機能していることが明らかになった．

種子の登熟期及び発芽生長期にかけてのVPEの活性とタンパク量の変動

ヒマ種子の胚乳細胞におけるVPEのタンパク量の変動について，特異抗体を用いたイムノブロット解析により調べた（図6 a）．登熟初期及び発芽8日目の組織ではプロセシング活性が低かった．これを支持するようにバンドが殆ど検出されず，酵素量が少量であることが考えられた．また，乾燥種子における酵素量は活性から予想されるより多かった．カボチャ種子内のプログロブリンをプロセスする活性が，乾燥種子では検出されなかったという報告がある (Hara-Nishimura and Nishimura, 1987)．これらの結果から考えると乾燥種子のプロセシング酵素は不活性化されているか，あるいはまた，活性を持つプロセシング酵素は乾燥種子から抽出されにくいのかもしれない．

先に乾燥ヒマ種子から精製されたVPEは37 kDaであることが示されている(Hara-Nishimura et al., 1991)．37 kDaのバンドは登熟及び発芽種子の胚乳から検出された．一方，41 kDaのバンドが発芽2日目まで検出され，イムノブロット解析に用いる抗体をmaltose-binding proteinとVPEの融合タンパク質に対する抗体に変えても，同様の結果が得られた．

VPEはプログロブリンのプロセシングサイトを含む10アミノ酸残基

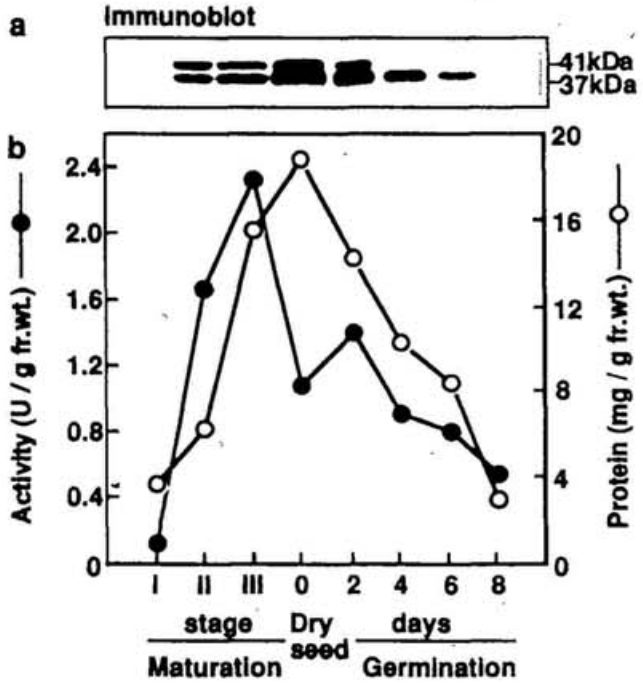


図６．ヒマ種子の登熟期 (Maturation) から乾燥期 (Dry seed) 及び発芽生長期 (Germination) にかけてのVPEのタンパク量の変動及び活性の変動 (a) 各ステージのヒマ種子胚乳からのタンパク抽出液をSDS-PAGE後，抗VPE抗体を用いてイムノブロット解析を行った．(b) 各ステージの抽出液中に存在する合成ペプチドを切断するVPE活性及び抽出液のタンパク質量をそれぞれ黒丸及び白丸で示す．登熟ステージI，II，III はそれぞれ登熟前期，中期，後期，0は乾燥種子，発芽生長期2，4，6，8はそれぞれ播種後の日数を示す．

を含む合成ペプチドのアスパラギン残基のC末端側のペプチド結合を切断する活性を持つ(Hara-Nishimura et al., 1991; Hayashi et al., 1988). ヒマ種子protein-storage vacuoleからの粗抽出液から，この合成ペプチドを2つのペプチドに分解するプロセシング活性を持つが，それ以外のペプチドの分解は起こらないことが示されている(Hara-Nishimura et al., 1991)．つまり，protein-storage vacuole内にはVPE活性以外に，このペプチドを分解するプロテアーゼは存在しないことが示唆される．そこでこの基質を用いて登熟期から発芽生長期の各ステージのヒマ種子胚乳の粗抽出液からVPE活性検出を行った．プロセシング活性はヒマ種子の登熟に伴い，胚乳での液胞プロセシング活性の蓄積が全タンパク質の蓄積に先がけて起こった（図6b）．全タンパク質量の増加は種子タンパク質の生合成に伴って起こり，乾燥種子で最大になる．発芽生長期には4日以降の胚乳細胞は老化していき，10日後には胚乳は子葉に吸収されて消失する．全タンパク質量も発芽に伴って減少していく．それに対してプロセシング活性は登熟後期に最大になり，種子の発芽に伴って減少していった（図6b）．

*In vitro*での種子タンパク質前駆体のプロセシング

精製AP及びVPEが液胞タンパク質のプロ型前駆体をプロセスすることができるかどうかを調べた．登熟中期のヒマ種子胚乳を[^{35}S]メチオニンでパルス標識し，ショ糖密度勾配遠心により分画しプロ型タンパク質を含むER画分を調製し，パルス標識後チェイスしたヒマ胚乳からprotein-storage vacuole画分を調製した．Protein-storage vacuole画分には2Sアルブミン，11Sグロブリンの小サブユニットや，RCA，リシンが含まれていた（図7，lane 1）．これに対してER画分には2Sアルブミンや11Sグロブリン，RCAとリシンの3つのプロ型前駆体が含まれていた（図7，lane 2）．標識されたプロ型タンパク質を*in vitro*で

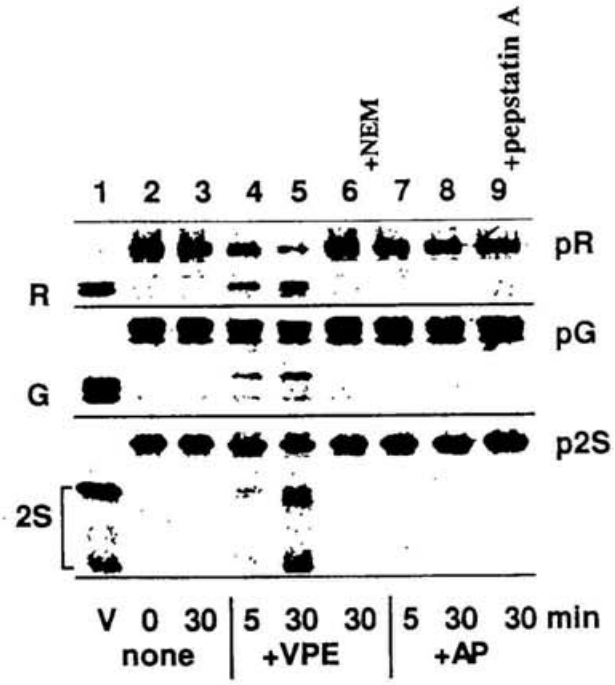


図７．登熟ヒマ胚乳から調製したER画分に局在する前駆体タンパク質の*in vitro*でのプロセシング

[^{35}S]メチオニンと[^{35}S]システインで１時間パルス標識したヒマ登熟中期の胚乳からショ糖密度勾配遠心で粗面小胞体画分(ER)を，同じく１時間のパルス標識後に２時間メチオニンとシステインでチェイスした胚乳から液胞画分 (V)を調製した．lane 1；成熟型種子タンパク質（2Sは2Sアルブミン，Gは11Sグロブリン，RはRCAとリシンを示す）を含む液胞画分．lane 2；前駆体タンパク質（p2Sはプロ2Sアルブミン，pGはプロ11Sグロブリン，pRはプロRCAとプロリシンを示す）を含むER画分．lane 3；ER画分を酵素なしで37℃ 30分反応させた反応溶液．lanes 4, 5；ER画分に0.4 mUのVPEを加えて37℃ 5分 (lane 4) 30分 (lane 5) 反応させた反応溶液．lane 6；ER画分にVPEとその活性阻害剤であるN-エチルマレイミド (NEM)を加えて 30分反応させた反応溶液．lanes 7, 8；ER画分に0.4 mUのAPを加えて37℃ 5分 (lane 7)， 30分 (lane 8) 反応させた反応溶液．lane 6；ER画分にAPとその活性阻害剤であるペプスタチンAを加えて 30分反応させた反応溶液．各画分及び反応溶液をSDS-PAGE後，フルオログラフィー処理した．

のプロセシング活性測定のための基質として用いた．酵素を加えずにこのER画分を反応させてもプロ型タンパク質のプロセシングは起こらない（図7，lane 3）．このことからプロ型タンパク質のプロセシングを行うVPEは，ER内では不活性型として存在するのと同様にAPも不活性型前駆体として存在すると考えられる．

すでにヒマVPEがカボチャ種子に含まれる複数の前駆体タンパク質のプロセシングを行うことが示されている (Hara-Nishimura et al., 1982)．ヒマから調製したER画分にVPEを加えて30分間反応させると，3つすべてのプロ型タンパク質が減少するとともに，液胞内の成熟型タンパク質と同じサイズのバンドの増加が起こった（図7，lanes 4, 5）．この3つの前駆体のプロセシングはVPEの阻害剤である2 mM NEMによって阻害された（図7，lane 6）．これに対してAPを加えた場合には変化は見られず，プロセシングは起こらなかった（図7，lanes 7-9）．以上の結果から前駆体タンパク質のプロセシングはAPではなくVPEによって起こることが示された．

APによる*in vitro*でのプロ2Sアルブミンの内部のプロペプチドのプロセシング

2Sアルブミン前駆体は成熟型タンパク質の2つのサブユニットの間にプロペプチドを持つことが報告されている．そのペプチドは生合成の間に切断されると考えられる．上記の結果及びHara-Nishimura et al. (1991) の報告が示すようにVPEによるプロセシングはかなり速やかに起こる．このことを考えると，2Sアルブミン前駆体はVPEによるプロセシングを受けた後，分解されるべきプロペプチドの除去が他のプロテアーゼによって起こることが予想される．

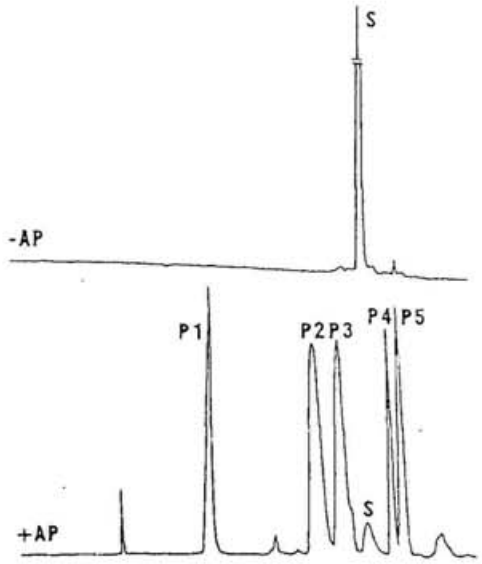
このプロペプチドの除去にAPが関与しているかどうかを調べるために，2Sアルブミンの内部のプロ領域を含んだペプチドの*in vitro*での分解を行った．シロイヌナズナ2Sアルブミン-2は成熟型タンパク質の

一次構造とcDNAから予想される前駆体タンパク質のアミノ酸配列との比較から，翻訳後のプロセシングがどのサイトで起こるかについて明らかになっている(Krebbers et al., 1988)．そこでこのシロイヌナズナプロ2Sアルブミン-2前駆体の内部のプロペプチドの配列を含んだ18アミノ酸残基からなるペプチドを合成し，APの基質として用いた（図８c）．合成ペプチドに酵素を加えて30分間 pH 3.0の条件で反応させると，そのプロペプチドが減少し代わって５つのペプチドフラグメントが得られた（図８a）．それぞれのペプチドフラグメントのアミノ酸組成について調べた結果，APは３カ所でそのペプチドを切断したことが判った（図８b, c）．まずはじめの切断はフェニルアラニン－7とアスパラギン酸－8の間で起こり，P2とP5のペプチドを生じた．2番目の切断はロイシン－9とグルタミン酸－10の間で起こり，P3とP4のペプチドを生じた．3番目の切断はアスパラギン酸－12とイソロイシン－13の間で起こりP1ペプチドを生じた（図８ c）．これはP4やP5の分解産物であると考えられる．この結果はAPが，疎水的アミノ酸を認識してそのペプチド結合を切断することを示唆している．APはVPEによるプロセシングによって2つのサブユニットが生じた後のプロペプチドの領域の分解に関与しているのかもしれない．しかしAPによる合成ペプチドの切断はヒマ液胞内のpHと考えられているpH5.5の条件では起こらなかった．図９ではAPとVPEの活性へのpHによる影響を示している．APのプロペプチドを切断する活性はpH 4以下で検出され，その至適pHは3.0だった．これに対してVPEの活性はpH 3.0では検出されず，至適pHは5.5だった．この結果はAPの活性はprotein-storage vacuole内では非常に低いことが示唆され，プロペプチドの切断はかなり遅い速度で起こっていると考えられた．

ヒマの各器官でのVPEの局在

ヒマの種子胚乳以外の子葉，胚軸，根，本葉におけるプロセシング

(a)



(b)

	Amino acid composition (mol)					
	S	P1	P2	P3	P4	P5
Asp	6.20 (6)	1.11 (1)	2.39 (2)	3.39 (3)	2.89 (3)	4.08 (4)
Ser	0.92 (1)	n. d.	0.97 (1)	0.84 (1)	n.d.	n.d.
Glu	4.26 (4)	1.97 (2)	1.24 (1)	1.02 (1)	2.89 (3)	2.44 (3)
Pro	2.00 (2)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Gly	1.05 (1)	1.08 (1)	n. d.	n. d.	0.88 (1)	1.05 (1)
Ile	2.08 (2)	0.85 (1)	n. d.	n. d.	0.85 (1)	0.82 (1)
Lue	2.08 (2)	n. d.	1.05 (1)	2.09 (2)	0.29 (0)	1.05 (1)
Phe	1.02 (1)	n. d.	1.02 (1)	1.05 (1)	n. d.	n. d.

(c)

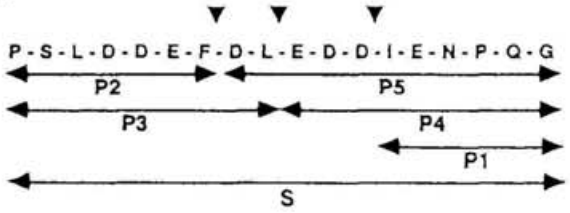


図８．アラビドプシス2Sアルブミンの内部のプロペプチド由来の合成ペプチドのAPによる切断

(a) 合成ペプチド (S) と精製APによる切断によって生じた5本のペプチドフラグメント (P1からP5) のキャピラリー電気泳動のパターン．(b) (a) の各ピークのアミノ酸組成をプロリンを基準にしたモル比で示す．n. d. は検出されていないことを示す．(c) 合成ペプチドのアミノ酸配列及び(b)から予想されるAPによる切断部位を黒三角で示す．

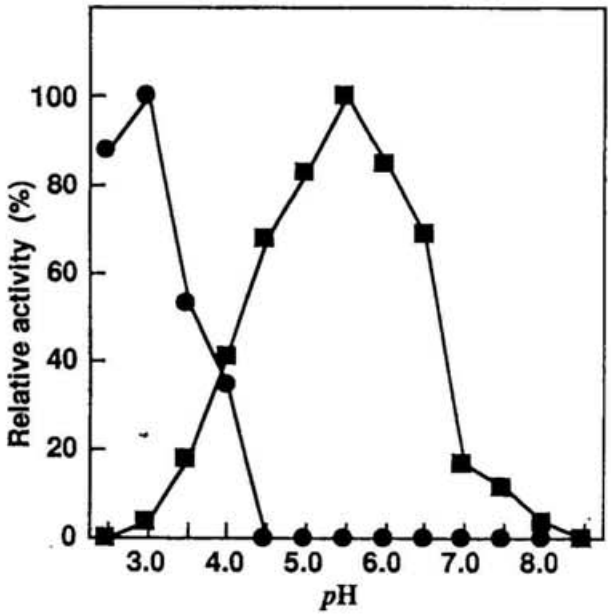


図９．精製ヒマAP及び精製ヒマVPEの至適pHの比較
黒丸はAP活性を，黒四角はVPE活性を示す．合成ペプチドを基質として各精製酵素を用いて37℃ 30分間反応させたあと，キャピラリー電気泳動で解析した．

酵素の存在について調べた．各器官1 gあたりのプロセシング活性で比較した（図１０）．胚乳における活性の約30%の活性が登熟期の子葉から検出された．この結果はイムノブロット解析による結果からも支持された（図１０a, b）．胚軸，根及び本葉のような栄養器官からは胚乳における活性の0.2%から0.8%の活性が検出された（図１０b）．イムノブロット解析ではタンパクを検出することができなかった（図１０a）が，各抽出液を5倍量に増加するとバンドが検出された．37 kDaと41 kDaの2本のバンドは子葉の場合も胚乳の時と同様に検出された．これらの結果から，VPEは胚乳のような貯蔵器官だけでなく，栄養器官にも存在することが示された．

様々な植物器官からのVPE活性の検出

様々な植物におけるプロセシング活性が存在するかどうかについて調べた．カボチャ及びダイズの登熟種子の子葉からはヒマ胚乳の25%から32%の活性が検出された（図１１）．単子葉であるコメの登熟種子からもヒマ胚乳の5%未満の低い活性ではあるが検出された(図１１)．さらにヤエナリやホウレンソウの胚軸，根，本葉のような栄養器官でもヒマの場合と同様に低い活性が検出された（図１１）．これらの結果からプロセシング活性は貯蔵器官では高く，栄養器官では低いことが示唆された．VPEは貯蔵器官の種子タンパク質だけではなく，本葉，根及び胚軸のような栄養器官での液胞タンパク質のプロ型前駆体のプロセシングに関わっていると考えられる．

VPEの細胞内局在性

VPEの細胞内局在性について明らかにするため，特異抗体を用いて登熟ヒマ種子胚乳の免疫電顕観察を行った．その結果，protein-storage vacuoleのマトリクス及び直径300 nmのデンスベシクル上に本酵素の局在を示す金粒子が検出された（図１２ A, B, C）．本酵素の

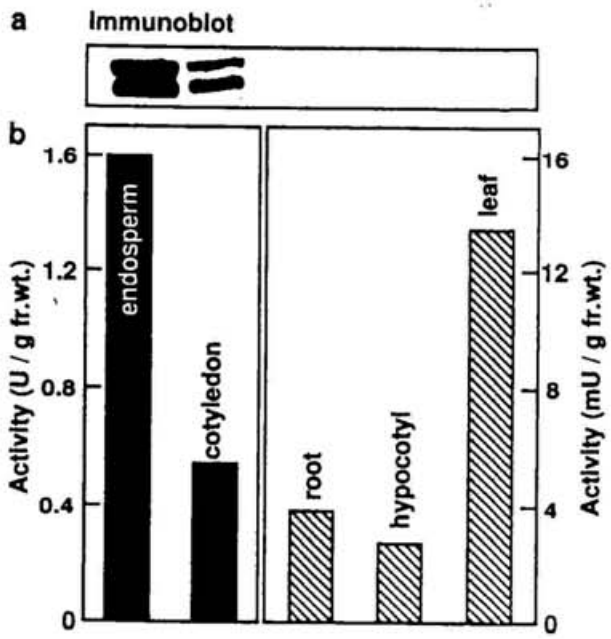


図１０．ヒマの各器官のVPE活性

(a)ヒマの胚乳 (endosperm)，子葉 (cotyledon)，根 (root)，胚軸 (hypocotyl) 及び本葉 (leaf) からのタンパク抽出液をSDS-PAGE後，抗VPE抗体を用いてイムノブロット解析を行った．(b) 胚乳，子葉からのタンパク抽出液中のVPE活性を各器官１ｇあたりのユニット数で，根，胚軸及び本葉からのタンパク抽出液中のVPE活性を各器官１ｇあたりのミリユニット数で示した．

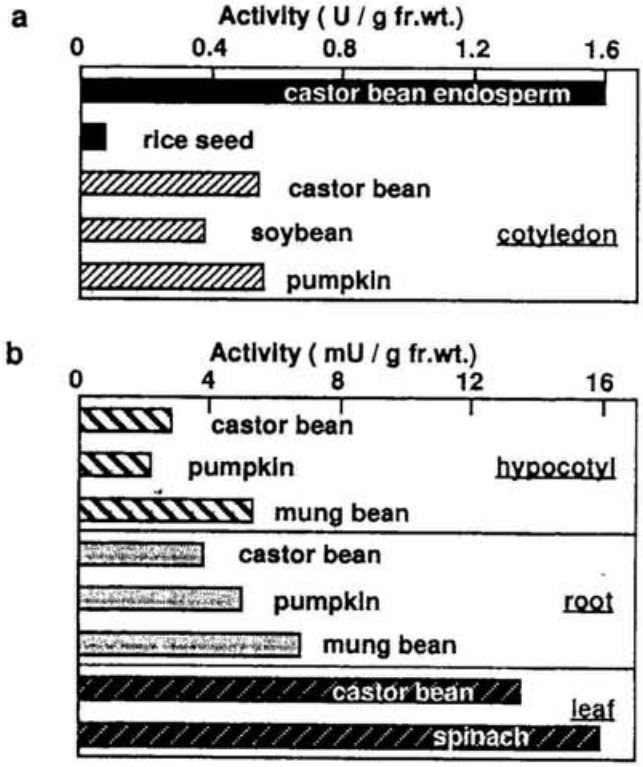


図１１．様々な植物の各器官におけるVPE活性

(a) ヒマ胚乳，イネ種子，ヒマ，ダイズ及びカボチャの子葉からのタンパク抽出液中のVPE活性を各器官１ｇあたりのユニット数で示した．

(b) ヒマ，カボチャ，ヤエナリの胚軸及び根，ヒマ，ホウレンソウの本葉からのタンパク抽出液中のVPE活性を各器官１ｇあたりのミリユニット数で示した．

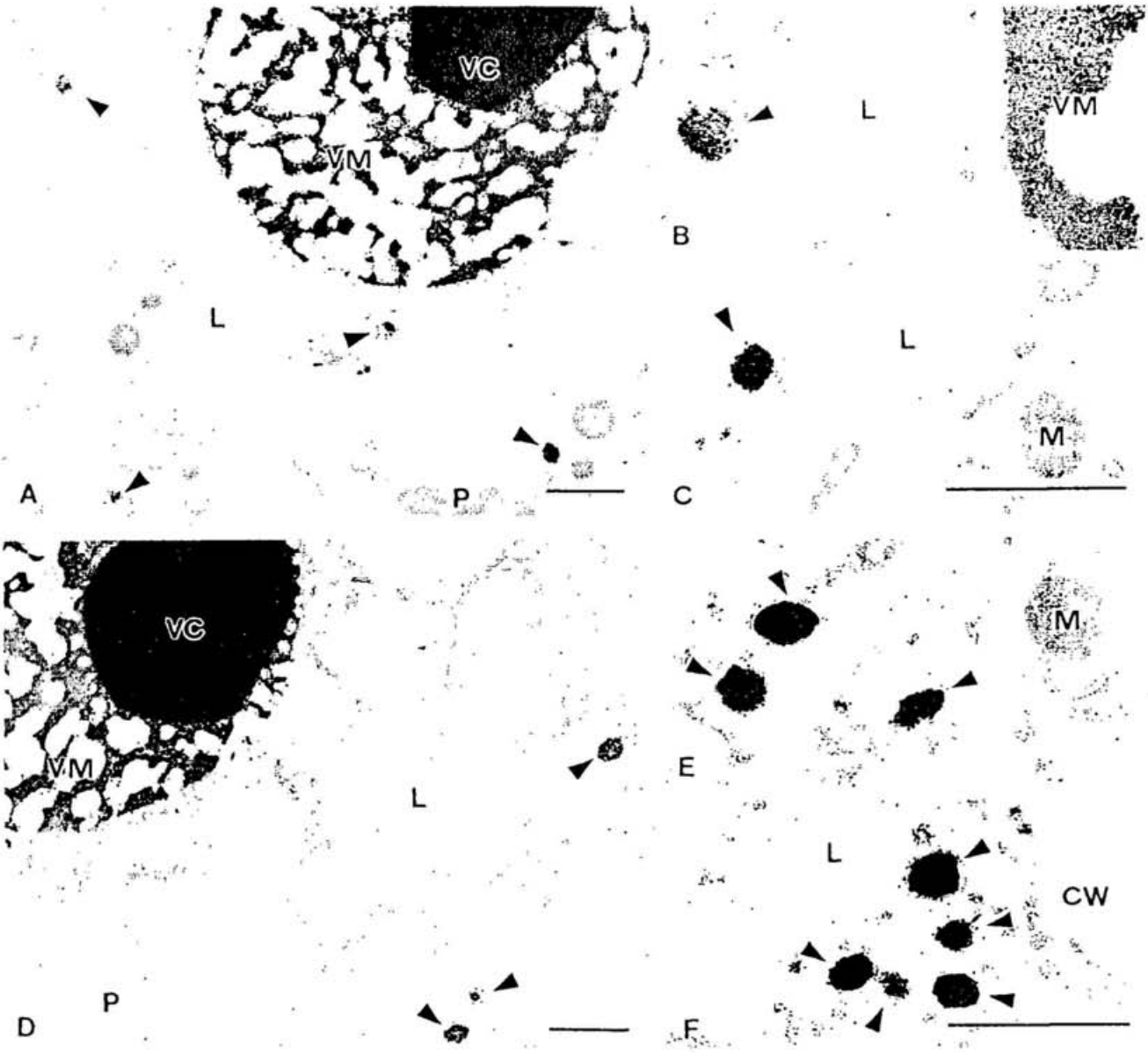


図１２．登熟ヒマ種子の胚乳細胞内のデンスベシクル中のVPEの局在抗VPE抗体 (A, B, C) 及び抗11Sグロブリン抗体 (D, E, F) を用いて登熟ヒマ種子胚乳の免疫電顕観察を行った．デンスベシクルは arrowheads で示す．VM, VC, L, M, P, CWはそれぞれ液胞可溶性マトリクス，液胞不溶性クリスタロイド，リピッドボディ，ミトコンドリア，プラスチド及び細胞壁を示す．バーは１μmである．

protein-storage vacuoleマトリクスにおける局在性は上記の分画実験の結果によっても支持される．一方デンスベシクルは直径50 nmのゴルジベシクルとは形態的に明らかに異なる．ヒマ種子胚乳を用いたパルスチェイス実験から主要タンパク質である11S グロブリンの前駆体がrERからデンスベシクルを経由し液胞へ輸送されることが示されている (Fukasawa et al., 1988)．この11S グロブリンに対する抗体を用いた免疫電顕でも液胞及びデンスベシクル上に金粒子が検出された(図１２D, E, F)．この結果からVPEも11S グロブリンと同様にデンスベシクルを経由して液胞に輸送されることが示唆された．

酵母細胞におけるヒマVPEの発現

ヒマVPEがデンスベシクルに局在すること及びそのcDNAを用いた解析 (Hara-Nishimura et al.,1993b, 1995)から，ヒマVPEの活性発現機構の存在が示唆されている．そこでこれについて解析するため,酵母細胞(*Saccharomyces cerevisiae*)での発現を行った．大腸菌と酵母のシャトルベクターで，2μM DNAの複製起点，*Gal1*プロモーター，*CYC1* 転写終結配列及び選択マーカーとして*URA3* 遺伝子を含む，発現ベクターpYES2の*Gal1*プロモーターの直下にヒマVPE前駆体をコードするcDNAを挿入したコンストラクトを構築した．酵母 (*Saccharomyces cerevisiae*) の正常 (*PEP4*) Ura^-株及び液胞プロテイナーゼA (PrA)を欠損した株 (*pep4*) Ura^-株にコンストラクトを形質転換し，ウラシル要求性で選抜した．Ura^+の表現型を示したコロニーをガラクトースを含む最小培地に移して培養し細胞を回収した．各形質転換酵母細胞の抽出液を抗VPE抗体を用いたイムノブロットで解析した（図１３）．その結果，ガラクトースで発現誘導した*PEP4*形質転換酵母株から59 kDa，46 kDaの２本のバンドが検出された（図１３，lane 2）．この２本のバンドはグルコースで培養した形質転換酵母の抽出液からは検出されなかった

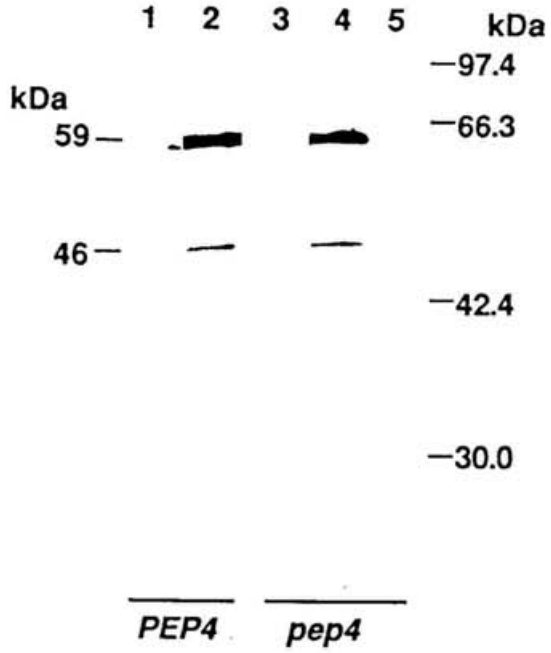


図１３．ヒマVPEの酵母 (*Saccharomyces cerevisiae*) での発現
発現ベクターにつないだヒマVPEを正常株 (*PEP4*; lanes 1, 2) 及びプロテイナーゼA欠損株 (*pep4*; lanes 3, 4) に導入した．各形質転換体をグルコース (lanes 1, 3) 或いはガラクトース培地 (lanes 2, 4) で培養しタンパク抽出を行い，各タンパク抽出液をSDS-PAGE後，抗VPE抗体を用いたイムノブロット解析を行った．検出された 59 kDa, 46 kDa タンパク質の位置を左側に示す．発現ベクターのみを導入しガラクトース培地で培養した酵母細胞 (*pep4*)からの抽出液からは抗VPE抗体に反応するバンドは検出されなかった (lane 5)．右側の数字はマーカータンパク質の相対質量 (kDa) を示す．

（図１３，lane 1）．また発現ベクターのみを導入した形質転換酵母細胞のガラクトースで発現誘導した細胞の抽出液からも検出されなかった（図１３，lane 5）．

*PEP4*遺伝子によってコードされるプロテイナーゼAは，プロテイナーゼA自身やプロテイナーゼBやCPYといった液胞内の様々な加水分解酵素の活性化に関与していると考えられている(Ammerer et al., 1986; Jones et al., 1982; Woolford et al., 1986)．このため*pep4*株では液胞内加水分解酵素による分解活性が非常に低い．46-kDaタンパク質が59-kDaタンパク質の分解産物である可能性が考えられたので，*pep4*形質転換酵母株の抽出液を用いてイムノブロットで解析した（図１３，lane 3, 4）．*pep4*形質転換酵母株からも*PEP4*株の
時と同様に59 kDa，46 kDaの２本のバンドが検出された（図１３，lane 4）．このことは46-kDaタンパク質は59-kDaタンパク質が酵母細胞内の加水分解酵素によって分解を受けてできたものではないことを示唆している．

<u>*pep*4株での59-kDaタンパク質から46-kDaタンパク質への変換</u>

VPEはrER上でプロ型前駆体として合成され，protein-storage vacuole内で成熟型に変換すると考えられる(Hara-Nishimura et al., 1993b)．59-kDaタンパク質及び46-kDaタンパク質がそれぞれVPEのプロ型前駆体及び成熟型酵素ではないかと考えられた．そこでガラクトース誘導培地内での反応の間59-kDaタンパク質から46-kDaタンパク質への変換が起こるかどうかについて調べた（図１４）．59-kDaタンパク質はガラクトース誘導1時間後の細胞から検出され，46-kDaタンパク質は3時間後の細胞から検出された（図１４）．59-kDaタンパク質はその後増加し3時間後に最大レベルに達しその後減少していった．この59-kDaタンパク質の減少は46-kDaタンパク質の蓄積と関連づけられた．以上の結果から59-kDa タンパク質が細胞内で経時的に46-kDa タンパク質に

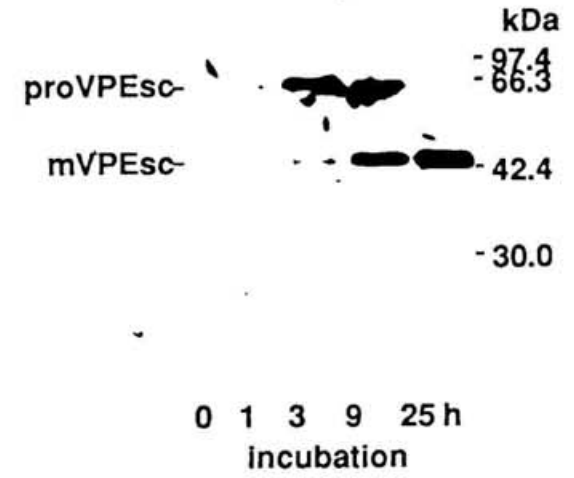


図１４．*pep4* 形質転換体中での 59-kDa VPE から46-kDa VPEへの変換
VPEを導入した*pep4* 株をガラクトース培地で1時間から25時間培養し，各培養時間の細胞からタンパク抽出を行い，抗VPE抗体を用いたイムノブロット解析を行った．左側の proVPEsc 及び mVPEsc はそれぞれ 59-kDa VPE 前駆体 及び46-kDa 成熟型 VPEを示す．右側の数字はマーカータンパク質の相対質量 (kDa) を示す．

変換されること及びその変換にはプロテイナーゼAが関与していないことを示している．つまり59-kDa タンパク質がプロ型前駆体，46-kDaタンパク質が成熟型であり，前駆体から成熟型への変換を示していると考えられた．

酵母細胞内でのヒマVPEへの糖鎖付加

酵母細胞に導入したヒマVPEのcDNAはN末端側にシグナルペプチドを持つ55 kDaのプレプロ型前駆体 タンパク質をコードしている．翻訳と同時にシグナルペプチドの切断を受けることにより，53 kDaのプロ型前駆体タンパク質がrERでできてくる (Hara-Nishimura et al., 1993b, 1995)．酵母で発現させたVPEは59-kDa タンパク質で，予想される53 kDaのプロ型前駆体より6 kDa大きかった．本酵素にはN結合型糖鎖付加サイトが2ヶ所ある．53 kDaのプロ型前駆体に糖鎖がついて59 kDaが生じることが考えられる．

59-kDa及び46-kDaタンパク質にN結合型糖鎖がついているかどうか調べるため，糖タンパク質からN結合型糖鎖を切断する酵素，*N*-グリコシダーゼF (Chu, 1986) を使って解析した．VPEをガラクトースで発現誘導をかけた*pep4*株の抽出液に*N*-グリコシダーゼFを加えて反応させ，その反応液をSDS-PAGEにかけ，抗VPE抗体及び抗CPY抗体を用いてイムノブロット解析を行った．CPYはN結合型糖鎖付加サイト4つ持っており(Valls et al., 1987)，そこに結合した糖鎖すべてをあわせると約10 kDaであると報告されている(Hasilik and Tanner, 1978)．CPYを糖鎖切断処理のマーカーとして用いたところ，酵素処理15時間後の抽出液からは61-kDaタンパク質（図１５a，lane 5）にかわって51-kDaタンパク質（図１５a，lane 6）が検出され，糖鎖除去が起こっていると考えられた．VPE59-kDa前駆体はガラクトースで1時間発現誘導かけた細胞から，成熟型46-kDaは25時間発現誘導かけた細胞から抽出した．

どちらのタンパク質も酵素を加えずに反応させた時には減少しな

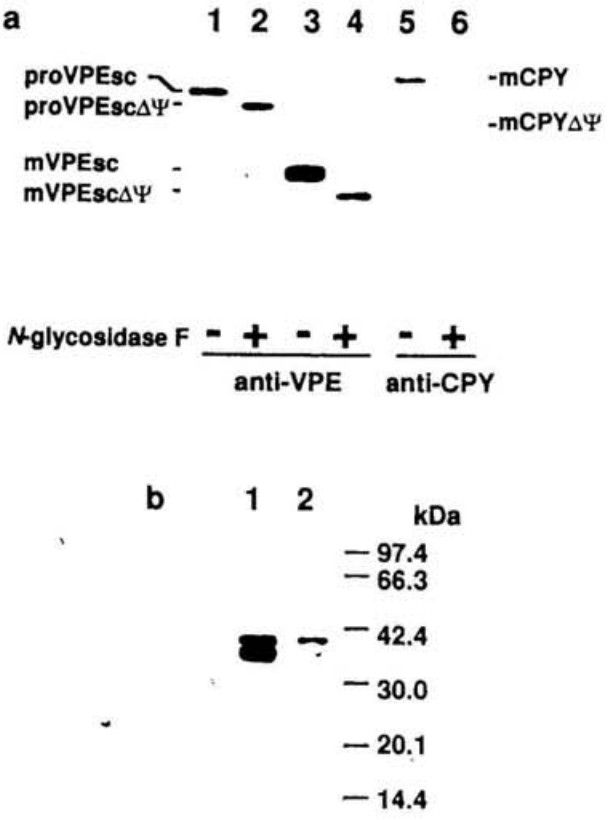


図１５．酵母で発現させたVPEの脱糖鎖処理

(a) VPEを導入した*pep4*株をガラクトース培地で1時間 (lanes 1, 2) 及び25時間 (lanes 3-6) 培養し，各細胞からの抽出液を*N*-グリコシダーゼFを加えずに (*N*-glycosidase F -; lanes 1, 3, 5) または*N*-グリコシダーゼFを加えた状態で (*N*-glycosidase F +; lanes 2, 4, 6) 37℃で15時間反応させた．その反応液をSDS-PAGE後，抗VPE抗体 (anti-VPE; lanes 1-4) 及び抗CPY抗体 (anti-CPY; lanes 5, 6)を用いてイムノブロット解析した．左側のproVPEscΔΨ 及び mVPEscΔΨ はそれぞれ 糖鎖除去された59-kDa VPE前駆体 及び46-kDa 成熟型 VPEを示す．mCPY 及び mCPYΔΨはそれぞれ成熟型 CPY及び糖鎖除去された成熟型 CPYを示す．(b) (a) の lane 4で脱糖鎖処理した反応液 (lane 2) 及びヒマ登熟種子胚乳からの粗抽出液 (lane 1) を抗VPE抗体を用いてイムノブロット解析を行った．右側の数字はマーカータンパク質の相対質量 (kDa) を示す．

かった（図１５a，lanes 1, 3）．これに対して酵素を加えて反応させるとともに減少し，かわって53-kDaタンパク質（図１５a，lane 2）と41-kDaタンパク質（図１５a，lane 4）が検出された．酵素処理1時間の反応液からは59-kDaタンパク質にかわって56-kDaタンパク質が検出された（データーは示さない）．酵素処理によるそれぞれのタンパク質の変化から，約3 kDaの2つの糖鎖が切断されていることによると思われる．このことはヒマVPEが2ヶ所のN結合型糖鎖の付加サイトをもつことと一致する．以上の結果から酵母で発現させたVPEにはN結合型糖鎖付加が起こっていることが示唆された．

酵母で発現させたVPEが植物と同様であるかどうかについて調べるため，ヒマ種子抽出液から検出されるVPEと糖鎖除去したタンパク質の大きさを比較してみた．ヒマ抽出液からは37-kDaタンパク質とともに41-kDaタンパク質がprotein-storage vacuoleマトリクス画分から検出されている（図５，図６）．これを比較したところヒマ抽出液中の41 kDaのバンドは糖鎖除去された41 kDaのバンドと同じゲル上で同じサイズであった（図１５b，lanes 1, 2）．加えてこの糖鎖除去した反応溶液から41-kDaタンパク質にかわって37-kDaタンパク質が検出された（図１５b，lane 2）．以上の結果から酵母で発現させたVPEは，糖鎖除去すると植物VPEと同じサイズであることが示された．

形質転換酵母細胞内でのVPEの局在性

59-kDaVPE前駆体，成熟型46-kDaタンパク質の酵母細胞内における局在性について調べるため，Horazdovsky and Emr (1993) の方法に従い，*pep4*形質転換細胞を遠心処理によりP13画分，P100画分，S100画分に分画した．酵母液胞タンパク質アルカリフォスファターゼはP13画分から検出される(Horazdovsky and Emr, 1993)．抗CPY抗体を用いたイムノブロット解析から，CPYもP13画分にのみ検出された（図１６，lane 3）．59-kDa VPE，46-kDa VPEともにP13画分からのみ検出された

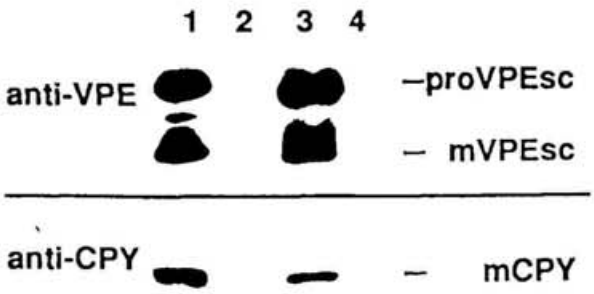


図１６．59-kDa proVPEsc及び46-kDa mVPEscの酵母細胞内での局在
VPEを導入した*pep4*株をガラクトース培地で15時間培養し，スフェロプラストにした後，遠心により細胞分画を行った．トータル画分 (lane 1)，S100画分 (lane 2)，P13画分 (lane 3)，P100画分 (lane 4)をSDS-PAGE後，上側は抗VPE抗体 (anti-VPE)，下側は抗CPY抗体 (anti-CPY)を用いてイムノブロット解析を行った．右側の proVPEsc 及び mVPEscはそれぞれ 59-kDa VPE 前駆体 及び46-kDa 成熟型 VPEを示す．mCPYは成熟型CPYを示す．

（図１６，lane 3）．培養液中からは両バンドともに検出されなかった（データーは示さない）．以上の結果から59-kDa，46-kDaタンパク質ともに酵母細胞内の液胞に局在することが示された．

CPYの成熟化には*PEP4*遺伝子によってコードされるプロテイナーゼAが関与しており，*pep4*株ではプロCPYは成熟型タンパク質へ変換されないことが知られている (Ammerer et al., 1986; Jones et al., 1982; Woolford et al., 1986)．今回ヒマVPEを導入し，発現させた*pep4*株の細胞からは成熟型CPYが検出された（図１５a，lane 5；図１６，lanes 1, 3）．この結果から酵母液胞内でVPEにより前駆体から成熟型への変換が起こっていることが示唆された．

形質転換酵母細胞からのVPE活性検出

形質転換細胞内で発現しているVPEが活性を持つかどうか調べるため，細胞内での本酵素の活性を測定した．VPEはプログロブリンのプロセシングサイトを含む10アミノ酸残基を含む合成ペプチドのアスパラギン残基のC末端側のペプチド結合を切断する活性を持つ(Hara-Nishimura et al., 1991; Hayashi et al., 1988)．このペプチドをプロセスするVPE活性はVPEを形質転換していない*PEP4*株，*pep4*株さらにベクターのみを形質転換した株からはともに検出されなかった（データーは示さない）．これに対してVPEを導入した*pep4*株からVPE活性が検出され，その活性はガラクトース誘導時間と共に増加した（図１７）．この活性の増加は46-kDaタンパク質の増加に伴っており，59-kDaタンパク質の量の変動とは一致していなかった．以上の結果から46-kDaタンパク質は植物VPEの活性型，59-kDaタンパク質は不活性型に相当すること，さらに細胞内で不活性型から活性型へ変換されることが示唆された．

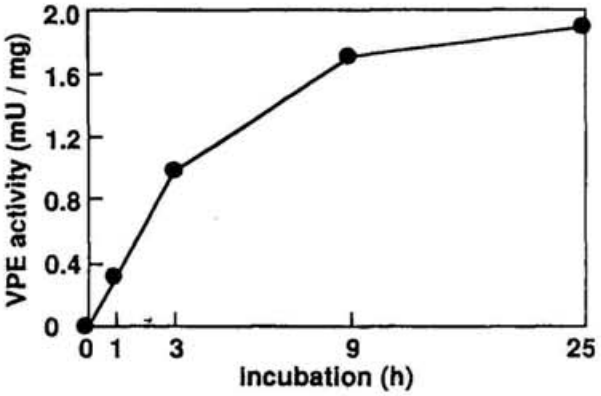


図１７．ガラクトース培地での培養によるVPE活性の増加
VPEを導入した*pep4*株をガラクトース培地で1時間から25時間培養し，各培養時間の細胞からタンパク抽出を行い，合成ペプチドを基質としてVPE活性を測定した．

酵母で発現させたVPEとヒマVPEとの比活性の比較

形質転換酵母で発現した成熟型46-kDa VPEとヒマ種子protein-storage vacuoleのVPEの比活性を比較した．図１８は，酵母及び精製VPE各0.04 mUをSDS-PAGE後抗VPE抗体を用いてイムノブロットした結果を示している．酵母での活性型46-kDa VPEとヒマ種子の精製37-kDa VPEのブロット上のシグナルはほぼ同程度であったことから（図１８，lane 1, 2），発現産物46-kDa VPEの持つ比活性はヒマ種子の酵素の比活性（約2 unit/mg protein）に匹敵することが明らかになった．

VPE発現による*pep4*株内のCPYの成熟化

さらに形質転換細胞内のCPYを指標としてVPEのプロセシング活性について調べた．酵母ではプロテイナーゼBやCPYのような液胞内加水分解酵素の成熟化及び活性化がプロテイナーゼAによって調節されていると報告されている(Ammerer et al., 1986; Jones et al., 1982; Woolford et al., 1986)．*PEP4*株からは成熟型CPYが検出される（図１９，lane 1）のに対して，*pep4*株からはプロ型前駆体の酵素が検出される（図１９，lane 2）．この*pep4*株にVPEを導入し発現させると，成熟型CPYが検出された（図１９，lane 3）．さらにこの抽出液から*pep4*株からは検出されないCPY活性も検出された（データーは示さない）．この酵素の変換は，ベクターのみを導入しガラクトース誘導をかけた細胞では起こらなかった（図１９，lane 4）．以上の結果から活性型VPEが酵母液胞内に存在し，プロテイナーゼAのかわりに前駆体CPYを成熟型に変換していることを強く示唆している．

CPY前駆体はアスパラギン残基のC末端側のペプチド結合の切断を受けて成熟型に変換することが報告されている (Valls et al., 1987)．先に示したとおり，VPEは分子表面のアスパラギン残基を認識し，そのC末端側のペプチド結合を切断する(Hara-Nishimura et al., 1993b)．酵母

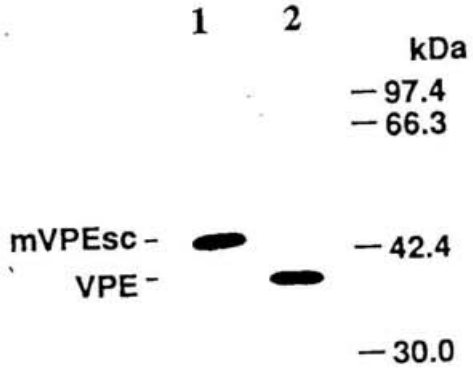


図１８．ヒマ種子胚乳中のVPEと酵母で発現させたVPEの比活性の比較
VPEを導入した*pep4*株をガラクトース培地で培養した細胞からタンパク抽出液 (lane 1)を，ヒマ種子胚乳から単離したprotein-storage vacuoleの可溶性マトリクス画分を調製した (lane 2)．VPE活性0.04mUを含んだ各抽出液をSDS-PAGE後，抗VPE抗体を用いてイムノブロット解析した．右側の数字はマーカータンパク質の相対質量 (kDa) を示す．

図１９．*pep4*酵母細胞内で発現させたVPEによるCPYの成熟化
VPEを形質転換していない正常株 (*PEP4*株；lane 1) 及びプロテイナーゼA欠損株(*pep4*株；lane 2)，VPEを形質転換した*pep4*株 (lane 3)，発現ベクターのみを形質転換した*pep4*株 (lane 4)からの抽出液をSDS-PAGE後，抗CPY抗体を用いてイムノブロット解析を行った．proCPY 及び mCPY はそれぞれ CPY 前駆体 及び成熟型 CPYを示す．

で発現させたVPEがプロCPYのアスパラギン残基も認識しプロセシングを行うことを示唆している．

変異VPEの酵母細胞内での発現

VPEはシステインプロテアーゼとして代表的なパパインをはじめとしたパパインファミリーとは殆ど相同性が見られない，新しいタイプのシステインプロテアーゼである(Hara-Nishimura et al., 1991; 1993b). パパインの活性中心近傍のアミノ酸配列とVPEホモログ間のアミノ酸配列 (Hara-Nishimura et al., 1993b; Shimada et al., 1994; Takeda et al., 1994; Kinoshita et al., 1995a, b; Alonso and Granell, 1995; Becker et al., 1995) との比較から，ヒマVPEの開始メチオニンから83番目のシステイン及び180番目のヒスチジン残基（N末端アミノ酸から26番目のシステイン残基及び123番目のヒスチジン残基）が活性中心であることが予想される（図２０）．そこでこれらの各アミノ酸残基をグリシン残基にかえたVPE (C83G, H180G)を作製し*pep4*株に導入した．この株をガラクトース誘導25時間行い，細胞の抽出液を用いて活性測定及び抗CPY抗体，抗VPE抗体を用いてイムノブロット解析を行った．合成ペプチドをプロセスするVPE活性は検出されず（データーは示さない），69 kDaのプロCPY及び59 kDaのVPE前駆体のみ検出された（図２１）．以上の結果は，今回解析した各アミノ酸残基が活性中心であること，さらに59-kDaタンパク質が自己限定分解によって46-kDaタンパク質になる可能性を強く示唆している．

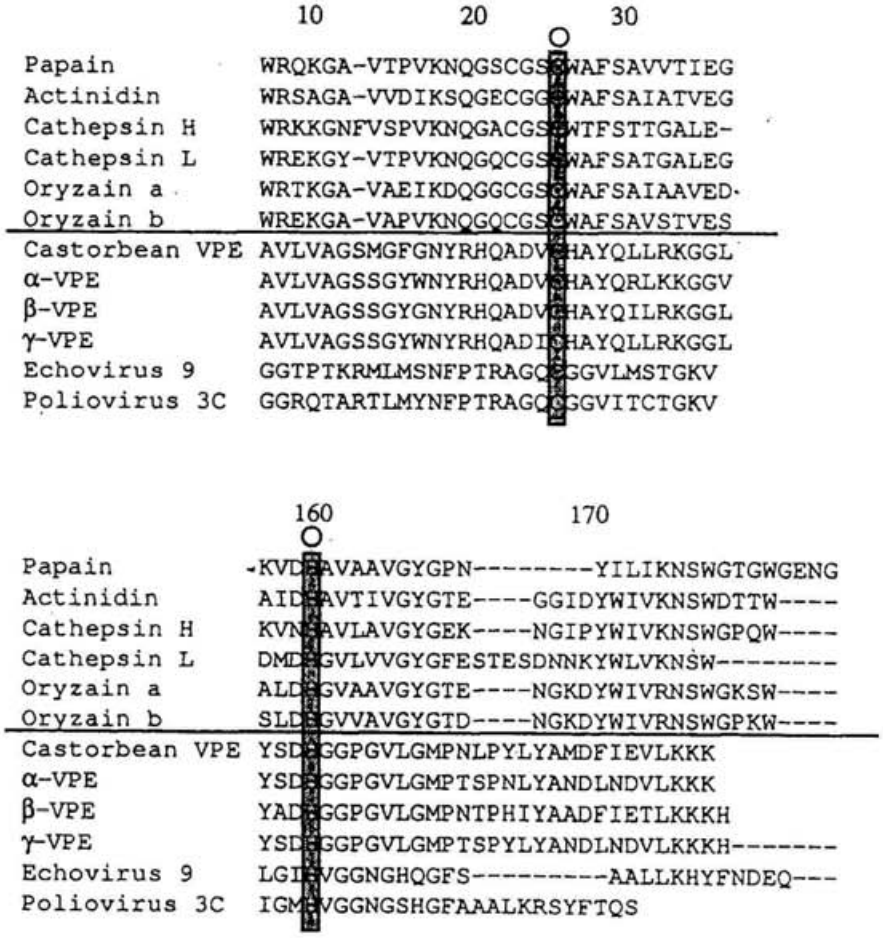


図２０．システインプロテアーゼの活性中心近傍のアミノ酸配列
数字はパパインを基準としたN末端からのアミノ酸残基数，○は活性中心の触媒部位を構成するアミノ酸残基を示す．

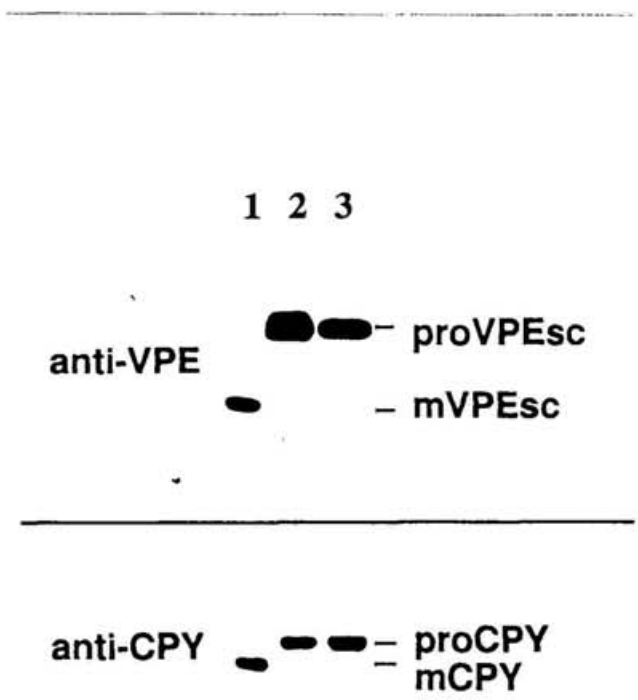


図２１．変異VPEの発現

正常VPE (lane 1) 及び変異VPE (C83G, lane 2; H180G, lane 3) を導入した *pep4*株をガラクトース培地で25時間培養し，各細胞からタンパク抽出を行った．各抽出液をSDS-PAGE後，抗VPE抗体 (anti-VPE) 及び抗CPY抗体 (anti-CPY)を用いてイムノブロット解析を行った． proVPEsc 及び mVPEsc はそれぞれ 59-kDa VPE 前駆体及び46-kDa 成熟型 VPEを，proCPY 及び mCPYはそれぞれ CPY 前駆体及び成熟型 CPYを示す．

考　察

VPEとAPによる種子タンパク質の成熟化

免疫電顕観察によりVPE及びAPがヒマの登熟種子のprotein-storage vacuoleのマトリクス領域に局在することが示された（図３，図５，図１２，Hara-Nishimura et al., 1993b）．種子の登熟期において両酵素のタンパク量は増加する（図４，図６）．このことから両酵素がともに種子タンパク質の成熟化に関与していることが示唆される．液胞に運ばれてきた種子タンパク質前駆体は，少なくともこの2つの酵素と接近するに違いない．

APの液胞タンパク質前駆体のプロセシング活性を検出するため，その基質としてER画分から調製したプロ型タンパク質群（プロ2Sアルブミン，プログロブリン，プロリシン及びプロRCAを含む）を使用した．精製VPEはこれら全ての前駆体タンパク質を成熟型に変換する（図７，lane 4, 5）．精製APを加えても前駆体タンパク質から成熟型への変化は見られなかった（図７，lanes 7, 8）．D'Hondt et al. (1993) はナタネのAPはpH 3.5の条件でシロイヌナズナのプロ2Sアルブミン−2をプロセスすると報告している．その基質はrabbit reticulocyte lysateを用いて*in vitro*で合成させたものである．そしてその合成産物をTCAを使って精製していることから，この基質が酸変性している可能性は否定できない．さらに彼らは酵素反応をpH 3.5という液胞内のpHよりかなり低い条件で行っている．そのため彼らの実験系が*in vivo*を反映しているかどうか疑問である．

登熟カボチャ種子から単離したprotein-storage vacuoleはVPE (Hara-Nishimura and Nishimura, 1987) 及びAP（データーは示さない）を含んでいる．このprotein-storage vacuole画分からもプログロブリンを成熟型に変換する活性が検出されている (Hara-Nishimura and Nishimura, 1987)．この活性はAPの阻害剤であるペプスタチンAによっては

阻害されず，N-エチルマレイミドやp-クロロメルクリ安息香酸といったチオール試薬によって阻害されることが示されている(Hara-Nishimura and Nishimura, 1987)．この結果はシステインプロテアーゼであるVPEが前駆体タンパク質の成熟化を行うことを示している．

カボチャ登熟種子のパルスチェイス実験により，前駆体タンパク質のプロセシングが液胞に輸送された後，速やかに起こることが示されている(Hara-Nishimura et al., 1993a)．ヒマ登熟種子から精製したVPEはプログロブリンやプロ2Sアルブミンを*in vitro*でも30分以内で成熟型に変換する高い活性を持つ (Hara-Nishimura et al., 1991)．このことからVPEによるプロセシングはプロペプチドの分解より先がけて起こると考えられる．

VPEはプロ2Sアルブミンの分子表面にある2つのアスパラギン残基を認識し，そのC末端側のペプチド結合を切断する(Hara-Nishimura et al., 1993a)．シロイヌナズナのプロ2SアルブミンもVPEによってプロセシングを受け，N末端側のプロペプチド，16アミノ酸残基からなるプロペプチドがついた小サブユニット，及び大サブユニットの3つのドメインになると考えられる(Krebbers et al., 1988; Hara-Nishimura et al., 1993a)．精製APは小サブユニットについたプロペプチドの部分の合成ペプチドを3つのサイトで切断した（図８）．この結果はAPがプロ2Sアルブミン-2の内部のプロペプチドの切断や分解に関与している可能性を示している．今回の研究から，APは基質のアミノ酸残基の片側に疎水性アミノ酸残基があるとそのC末端側を切断するが，親水性アミノ酸残基間のペプチド結合は切断しないことを示している（図８b, c)．これはオオムギのAPの性質(Kervinen et al., 1993) 及びウシのAP (Schwabe et al., 1993) がアスパラギン酸残基とチロシン残基の間，及び疎水性アミノ酸残基間のペプチド結合を切断するという報告とも一致する．これに対してナタネのAPは今回用いたものと同様の合成ペプチドのアスパラギン酸とアスパラギン酸の間を切断し，ロイシンとグル

タミン酸の間やアスパラギン酸とイソロイシンの間は切断しなかった(D'Hondt et al., 1993). APの基質特異性はVPEほど厳密でないことから，プロペプチドの分解に向いていると思われる．つまりプロ2Sアルブミンは基質特異性の非常に厳密なVPEによって切断を受けて，その後APによる分解によって，完全な成熟型タンパク質となることが考えられる．

さらに考えなければいけないのはAPの至適pHが3.0で，合成ペプチドを切断する活性がpH 4.0以上では非常に低かったことである（図９）．Nishimura (1982) はヒマ胚乳の液胞内のpHが5.0であることを報告した．このことはAPがprotein-storage vacuole内では最大の活性を発現することができないことを示している．2SアルブミンはVPEによるプロセシングを受けることにより，内部のプロペプチドが持った中間体として蓄積し，APによってトリミングを受けることによって成熟型になることが考えられる．

様々な植物器官でのVPE及びAPによる液胞タンパク質のプロセシングの系の存在

最近，貯蔵器官を中心に様々な植物器官からVPEのcDNAが単離されてきている(Hara-Nishimura et al., 1993b; Shimada et al., 1994;Takeda et al., 1994; Alonso and Granell, 1995; Becker et al., 1995). また，シロイヌナズナのVPEの3つのゲノミッククローン(αVPE, βVPE, γVPE)は，それぞれ器官特異的に発現していることが示された(Kinoshita et al., 1995a, b). βVPEは種子及び花粉特異的に発現し，αVPEやγVPEは栄養器官特異的に発現する(Kinoshita et al., 1995a, b). さらに，トマトの葉(Graham et al., 1985), タバコの柱頭 (Atkinson et al., 1993) に存在するプロテアーゼインヒビター，パパイヤのパパイン (Cohen et al., 1986) 及びタバコの本葉や培養細胞のキチナーゼ (Sticher et al., 1993)もアスパラギン残基のC末端側で翻訳後のプロセシングを受ける．

VPEと同様にAPもまた様々な植物器官に検出されている(Törmäkngas et al., 1994)，また今回の結果からヒマAPにも2つのアイソフォームがあることが示された．さらにイネのAPは異なった2つのcDNAが単離されている．Paris et al. (1996) は１つのAP前駆体がvegetative vacuoleへの輸送シグナルをN末端側プロ領域に，protein-storage vacuoleへの輸送シグナルとしてタンパク質内部のプロ領域に持つことを考察している．このことからAPもVPE同様2つ以上のホモログが存在すると思われる．AP及びVPEが，高等植物の種子や栄養器官に広く存在し，様々な液胞タンパク質の成熟化に関与していることが予想される．

VPEの細胞内輸送及び不活性型前駆体の存在

種子タンパク質のプロ型前駆体の細胞内輸送は[^{35}S]メチオニンでパルスラベルした登熟種子の子葉や胚乳を用いた細胞分画による実験で調べられている．カボチャの11Sグロブリン (Hara-Nishimura et al., 1985), 2Sアルブミン (Hara-Nishimura et al., 1993a)，ヒマ11Sグロブリン (Fukasawa et al., 1988) のプロ型前駆体はrERから密度1.24 g / cm^{3}であるデンスベシクルを経由して液胞へ輸送される．ヒマ11Sグロブリンに対する特異抗体を使っての免疫電顕観察から直径約300 nmのデンスベシクルに11Sグロブリンが含まれることが示された（図１２）．すでにカボチャの プロ2Sアルブミンもヒマで見られるデンスベシクルと同様のベシクルに蓄積されることが示されている(Hara-Nishimura et al., 1993a)．登熟カボチャ子葉から単離したデンスベシクルは様々なプロ型タンパク質を多く含んでいる(Hara-Nishimura et al., 1993a)．図１２の免疫電顕観察からVPEが他のプロ型タンパク質とともにベシクルに存在していることが示された．ところが，これらのデンスベシクルではプロ型タンパク質のプロセシングは起こらなかった(Hara-Nishimura et al., 1985)．このことからVPEは不活性型としてベシクルに局在していると考えられる．

VPEに対する特異抗体を使って行ったイムノブロット解析では2本のバンドが検出された．1つは精製された酵素と同じ37 kDaのバンドで，もう1つは41 kDaのバンドである（図５，図６）．大腸菌で発現させたmaltose-binding proteinとVPEとの融合タンパク質に対する抗体を使ってのイムノブロット解析でもこの2本のバンドは同様に検出された．このことから41 kDaのバンドも本酵素に関係していることが示唆される．単離されたヒマVPEのcDNAクローンは約55 kDaの前駆体をコードしている．N末端のシグナル配列を除くと，プロ型前駆体は約53 kDaであると予想され，これが自己分解または他のプロテアーゼによる加水分解を受け，41 kDaや37 kDaの成熟型になると考えられる(Hara-Nishimura et al., 1993b)．先に述べたように，液胞タンパク質の前駆体が様々な種子タンパク質のプロ型前駆体とともにデンスベシクル内に存在していると考えられるが，このベシクル内ではプロセシングは起こらない．このことからも53 kDaの前駆体は不活性型であると考えられる．この前駆体の自己分解または他のプロテアーゼによる加水分解を受けることが，活性化に関与していると考えられる．

酵母で発現させたVPEの生合成及び細胞内輸送

VPEのcDNAを用いた解析から，本酵素はシグナルペプチド，N末端プロペプチド，成熟型ポリペプチド及びC末端プロペプチドからなるプレプロ型前駆体として合成される (Hara-Nishimura et al., 1993b, 1995) と考えられている．酵母細胞内で発現させたVPEの酵母細胞内での局在性及び翻訳後に起こるプロセシングが植物液胞内と同様に起こっているかどうかについて検討する必要がある．図１６で示すように59-kDa及び46-kDaタンパク質がともに酵母細胞のP13画分に蓄積した．このP13画分には可溶性液胞タンパク質であるCPYのほとんどが検出された．また植物液胞膜タンパク質の１つであるα-TIPを同じ酵

母株で発現させると，α-TIPはP13画分に検出された．蛍光抗体法によるこの形質転換体の免疫化学的な観察で，この膜タンパク質が確かに液胞膜に局在していることが確認されている(Inoue et al.,1997)．以上の結果からこのP13画分は液胞画分を多く含んだ画分であり，59-kDa及び46-kDaタンパク質ともに酵母の液胞に局在していると考えられた．

一方，図１４で示す結果から59-kDaタンパク質は前駆体に相当し，成熟型46-kDaタンパク質に変換されると考えられる．この結果は植物液胞内と同様に翻訳後のプロセシングが酵母液胞内でも起こっていると考えられる．しかし，植物細胞内からはプロ型VPEは検出できていない．このことは植物細胞の液胞内では前駆体から成熟型への変換が極めて急速に起こっていることが予想される．

CPYやプロテイナーゼAのような酵母液胞タンパク質が液胞へ輸送されるためのシグナルは，前駆体のプロペプチド内に存在すると考えられている (Johnson et al., 1987; Valls et al., 1990; Klionskyet et al., 1988). このうちCPYはそのN末端側プロペプチド内のGln-Arg-Pro-Leu (QRPL) 配列が液胞輸送シグナルであることが示されている (Valls et al., 1990)．この配列はプロテイナーゼAの一次構造上に見られないのと同様に，VPE前駆体の一次構造上にも見られない (Hara-Nishimura et al., 1995)．同様にサツマイモの液胞貯蔵タンパク質スポラミンをはじめとして様々な植物液胞タンパク質が，QRPL 配列を持たないにも関わらず，酵母液胞へ輸送されることが示されている (Matsuoka and Nakamura, 1992; Tague and Chrispeels, 1987; Yarwood et al., 1987)．今回酵母で発現させたVPEも液胞に輸送された．このことから液胞への輸送シグナルやそれに対するレセプターが複数存在し，液胞へのタンパク輸送が起こると考えられる(Rothman et al., 1989; Valls et al., 1990).

<u>酵母で発現させたVPEの成熟化と活性発現</u>

酵母で発現させたVPEには精製ヒマVPEには見られない糖鎖付加が

起こった．この糖鎖付加が起こった46-kDaタンパク質は植物VPEと同様の比活性を示した（図１６）．このことは酵素への糖鎖付加は活性には影響しないことを示している．

大腸菌で発現させたVPE前駆体はプロセシング活性を持たず，deletionを起こしたVPEはプロセシング活性を持つことが示されている(Hara-Nishimura et al., 1993b, 1995)．このことから本酵素の活性化には前駆体タンパク質のプロペプチドの除去が必要であると考えられている (Hara-Nishimura et al., 1993b, 1995)．今回の結果では59-kDaタンパク質が不活性型であることが強く示唆され，VPEの活性は形質転換した細胞の成長とともに，59-kDaタンパク質でなく46-kDaタンパク質量の増加と平行して増加した（図１４）．さらにVPEの活性中心と考えられるシステイン残基及びヒスチジン残基をグリシン残基に変えた変異タンパク質を発現させたところ，59-kDaタンパク質のみが蓄積し，VPE活性も検出されなかった（図２１）．以上の結果から酵母細胞内で発現させたVPEは，59-kDaタンパク質が不活性型，46-kDaタンパク質が活性型であることが強く示唆された．

糖鎖除去した反応溶液から検出された41-kDa及び37-kDaタンパク質は,登熟種子から調製した抽出液から検出されるバンドと一致した(図１５）．この結果は酵母細胞と植物細胞で同様のVPE前駆体のプロセシングが起こっていることを示唆している．41-kDaタンパク質はVPEの中間産物で，２段階のプロセシングが本酵素の自己分解による成熟化に役割を果たしていると考えられる．

VPEの成熟化の第一段階目のプロセシングはどこで起こっているのだろうか？41-kDaタンパク質のC末端切断部位と予想される付近にはアスパラギン残基は見られなかった．最近カラスノエンドウのVPEがアスパラギン酸残基のC末端側を切断する活性を持つことが報告された．合成ペプチドPro-Ser-Leu-Asp-Asp-Glu-Phe-Asp-Leu-Glu-Asp-Asp-Ile-Glu-Asn-Pro-Gln-Glyを基質としてprotein-storage vacuoleマトリクス

画分と反応させたところ，Asp-Asp-配列のC末端側を切断する活性が検出された（データーは示さない）．Asp-Asp-配列はヒマ，ナタマメ，ダイズ，カボチャ及びシロイヌナズナのVPEの予想されるC末端側切断部位付近に保存されている．ヒマ酵素では54から57番目及び429から430番目に見出されている．VPE前駆体が自己分解によってこの部位で切断して41-kDaタンパク質の中間体に変換されることが考えられる．

先にヒマVPEの成熟型37-kDaタンパク質への変換は，その分子表面に存在する374番目のアスパラギン残基のC末端側で切断を受けることによって起こる可能性が示されている．Abe et al. (1993) はナタマメ酵素はN結合型糖鎖付加が起こったアスパラギン残基のC末端側のペプチド結合を切断しないことを報告している．もし酵母細胞内で374番目のアスパラギン残基に糖鎖付加が起こっていると，自己分解によるこの374番目のアスパラギン残基のC末端側のペプチド結合の切断は起きないと思われる．37-kDaタンパク質は糖鎖除去した46-kDaタンパク質の反応溶液からのみ検出された．このことから，VPEが糖鎖除去によって露出した374番目のアスパラギン残基を認識し，そのC末端側のペプチド結合の切断を行ったと考えられる．この切断により41-kDaタンパク質が最終精製標品の37-kDaタンパク質に変換されると予想される．

ヒマの374番目のアスパラギン残基は様々なVPEホモログ間では保存されていない (Hara-Nishimura et al., 1995)．本酵素ホモログは一般に33-39 kDaであると報告されている(Abe et al., 1993; Becker et al., 1995; Hara-Nishimura et al., 1991; Shimada et al., 1994; Kembhavi et al., 1993)．最終的なプロセシングサイトがVPEホモログ間で多様であるという可能性が考えられる．

以上の結果から酵母液胞内ではVPEの成熟化が自己分解によって起こることが強く示唆された．つまり自己分解による59-kDaタンパク質のプロペプチドの除去が本酵素分子構造に変化を起こし，結果として活性化につながると考えられる．そしてこのことは植物種子において

protein-storage vacuoleに輸送されてきたVPE前駆体が液胞内で自己分解によって成熟型に変換されることを示唆している．今後，VPE成熟型タンパク質のN末端及びC末端アミノ酸残基の決定やVPE前駆体のプロセシングサイトと予想されるアミノ酸残基付近の合成ペプチドを用いた解析から，VPEの活性化についてさらに明らかになると思われる．

液胞内におけるVPEの活性化と種子タンパク質の成熟化のためのカスケード機構

酵母細胞内で発現したVPEの前駆体は糖鎖の付加が起こるなど植物細胞とは異なるタンパク質の修飾機構が働いてはいるが，植物細胞と同様に液胞へ細胞内輸送されて，成熟化と活性化が起こる．この活性化は，活性中心に変異を起こした発現産物からは検出されなかったことから，酵母液胞内ではVPEが自己限定分解によるプロペプチドの除去が活性化を引き起こしていることが判った．この結果は，植物の種子細胞内でも液胞へ輸送されてきたVPE前駆体が同様の機構で活性化され，この活性型酵素がグロブリンをはじめとする各種の種子タンパク質の前駆体のプロセシングに関与しているというカスケード機構の存在を示唆する結果となった．

酵母CPY前駆体はアスパラギン残基のC末端側で切断されて，成熟型酵素になることが知られている(Valls et al., 1987)．プロテイナーゼA欠損株ではCPY前駆体が蓄積し，成熟型への変換は見られない(Jones et al., 1982)．しかしこの株にVPEを導入し発現させると，前駆体ではなく成熟型CPYが蓄積し，CPY活性が検出された．発現させたVPEが活性を持ち，CPYの成熟化を行ったことを示唆している．この結果は種子のprotein-storage vacuoleで機能しているヒマVPEが，酵母細胞のlyticな液胞と同様と思われるvegetative vacuole内で液胞タンパク質の成熟化に関与することを示唆している．

今後の展望

上述の通りAP及びVPEは様々な組織のprotein-storage vacuoleやvegetative vacuoleに存在し，液胞の機能発現に関与してくると思われる．今後さらに種子以外の植物器官のアイソザイムの発現解析等により，これらのプロテアーゼの液胞の機能発現への関与が明らかにされるものと期待される．

APは低い至適pHを持つのにも関わらずprotein-storage vacuole中に存在する．このAPのホモログである動物のカテプシンDの至適pHも2から4であり他のリソゾーム酵素と比べると低く，その基質となるタンパク質について未だによく判っていない．APの酵素学的性質及びターゲットとなるタンパク質について，さらに検討する必要があると思われる．また，最近カテプシンDが，アポトーシスが起こる過程で活性発現し，細胞内不要産物の分解に関与する可能性を示す報告がされている(Deiss et al., 1996)．植物APも同様に植物細胞の不要産物を時期特異的，つまり細胞を分解する必要があるときなどに，積極的に関与しているかもしれない．

液胞タンパク質前駆体の性質がプロセシングにより大きく変わることは，インゲンマメのαアミラーゼインヒビターの活性がプロセシングによって発現するという報告 (Pueyo et al., 1993) からも示されている．このことからVPEによる液胞タンパク質前駆体の特異的なプロセシングは，液胞タンパク質の最終的な構造を決定する重要な現象であると考えられる．VPEのアイソザイムが広く植物器官に分布し，植物液胞内の様々なタンパク質の成熟化，老化の時に発現誘導されてくるシステインプロテアーゼや食害によって誘導されてくるプロテアーゼインヒビター等の活性発現の制御に役割を果たしていることが予想される．これらのタンパク質の活性発現制御へのVPEの関与について，今回成功した酵母細胞内でのVPEの発現系を用いてさらに検討していくことにより明らかになることが期待される．

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報文目録

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A Vacuolar Processing Enzyme in Maturing and Germinating Seeds: Its Distribution and Associated Changes during Development

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Proprotein precursors of vacuolar components are transported from endoplasmic reticulum to the dense vesicles, and then targeted to the vacuoles, where they are processed proteolytically to their mature forms by a vacuolar processing enzyme. Immunoelectron microscopy of the maturing endosperm of castor bean (*Ricinus communis*) revealed that the vacuolar processing enzyme is selectively localized in the dense vesicles as well as in the vacuolar matrix. This indicates that the vacuolar processing enzyme is transported to vacuoles via dense vesicles as does 11S globulin, a major seed protein. During seed maturation of castor bean, an increase in the activity of the vacuolar processing enzyme in the endosperm preceded increases in amounts of total protein. The enzymatic activity reached a maximum at the late stage of seed maturation and then decreased during seed germination concomitantly with the degradation of seed storage proteins. We examined the distribution of the enzyme in different tissues of various plants. The processing enzyme was found in cotyledons of castor bean, pumpkin and soybean, as well as in endosperm, and low-level processing activity was also detected in roots, hypocotyls and leaves of castor bean, pumpkin, soybean, mung bean and spinach. These results suggest that the proprotein-processing machinery is widely distributed in vacuoles of various plant tissues.

Key words: Castor bean — Proprotein processing — Protein body — Seed protein — Vacuolar processing enzyme — Vacuole.

Most proprotein precursors of seed proteins are processed post-translationally into the mature proteins in the vacuoles of the maturing seeds. However, a few investigations of vacuolar processing activity have been reported, and the processing mechanism in plant vacuoles has not yet been fully characterized. An activity that converts proglobulin to mature 11S globulin, a major seed protein, accumulates in the vacuoles of maturing pumpkin cotyledons (Hara-Nishimura and Nishimura 1987, Hara-Nishimura 1987). An activity that converts proricin to ricin is present in both maturing and dry seeds of castor bean (Harley and Lord 1985), and an activity that converts proglycinin to glycinin is found in maturing soybean seeds (Scott et al. 1992). We purified a processing enzyme (37 kDa) from castor bean endosperm that is capable of converting several proprotein precursors with a broad range of molecular structures into their respective mature forms (Hara-Nishimura et al. 1991).

Abbreviation: ER, endoplasmic reticulum.

Proprotein precursors of pumpkin 11S globulin (Hara-Nishimura et al. 1985, Hayashi et al. 1988a, Akazawa and Hara-Nishimura 1985), pumpkin 2S albumin (Hara-Nishimura et al. 1993a) and castor bean 11S globulin (Fukasawa et al. 1988) are transported from endoplasmic reticulum (ER) to dense vesicles and then to vacuoles. In the present study, we show that dense vesicles mediate transport of a precursor of vacuolar processing enzyme to the vacuoles.

Previously we reported that vacuolar processing enzyme can recognize exposed asparagine residues on the molecular surface and cleave the peptide bond on the C-terminal side of each asparagine residue to produce mature protein (Hara-Nishimura et al. 1993a). The sequences of precursors of proteinase inhibitors of tomato leaf and *Nicotina alata* stigma were reported by Graham et al. (1985) and Atkinson et al. (1993), respectively. They suggest that proteolytic processing of non-storage tissues such as leaf and stigma occurs on the C-terminal side of an asparagine residue in a similar manner to that in vacuoles

of storage tissues.

We discussed that such proteolytic processing is a universal event in vacuoles of various tissues and a similar enzyme to that we isolated from castor bean seeds is involved in the proprotein processing in different tissues (Hara-Nishimura et al. 1991). Characterization of a vacuolar processing enzyme in various plant tissues is required if we are to determine whether a processing mechanism similar to that found in storage tissues also functions in non-storage tissues, such as leaves, roots and hypocotyls. Here we report the developmentally associated changes in the activity of such a processing enzyme and the distribution of this enzyme in various plant tissues, including non-storage tissues.

Materials and Methods

Plant materials—Seeds of castor bean (*Ricinus communis*), mung bean (*Vigna radiata*) and pumpkin (*Cucurbita* sp. Amakuri Nankin) were planted at 25°C in the dark and allowed to germinate and grow for up to 8 days. Castor bean and pumpkin seeds were grown in coarse vermiculite after soaking in water overnight. Mung bean seeds were germinated on nets on water. The seedlings of desired ages were harvested and endosperms, cotyledons, roots and hypocotyls were used for experiments.

To obtain maturing seeds or leaves, castor bean seeds were grown in a greenhouse and other seeds, including those of pumpkin, rice (*Oryza sativa*), soybean (*Glycine max*) and spinach (*Spinacia oleracea* L. var. kyoho), were sown on a farm that belongs to our institute. The maturing seeds of castor bean were harvested at three stages of seed development; the early stage (designated stage I) was 15 days to 20 days after anthesis, middle stage (designated stage II) was 25 days to 30 days after anthesis and late stage (designated stage III) was 35 days to 40 days after anthesis. Maturing seeds of pumpkin, soybean and rice were harvested at the late stage of seed development. Leaves of castor bean and spinach plants were also used for some experiments.

Extraction of proteins—Each gram fresh weight of cotyledon or endosperm was homogenized with 2 ml of 10 mM Tris-HCl (pH 7.5) on ice. In the case of non-storage tissues, such as hypocotyl, root or mature leaf, each gram fresh weight of tissue was homogenized on ice with 0.5 to 2 ml of 0.1 M sodium acetate (pH 5.5) that contained 0.1 M dithiothreitol and 1 mM EDTA. The homogenates were centrifuged twice at 20,000 × *g* for 15 min at 4°C. The activity of the vacuolar processing enzyme and the protein content of each supernatant were determined. The protein content was analyzed with a Protein Assay Kit (Bio-Rad, U.S.A.).

Preparation and fractionation of protein bodies—Protein bodies were prepared from 5 g of dry castor bean seeds by a non-aqueous isolation method, as described previously (Hara-Nishimura et al. 1982). Both light-micrographic examination and assays of marker enzymes indicated that the isolated protein bodies were intact and that contamination by other cell organelles or cytoplasmic components was negligible. After lysis of the protein bodies in 1 ml of a hypotonic buffer solution, 10 mM Tris-HCl (pH 7.5), the homogenate was centrifuged at 100,000 × *g* for 20 min at 4°C to separate it into a soluble fraction and an insoluble crystalloid that was composed of the major seed protein, 11S globulin. Membranes of protein bodies were precipitated with the crystalloids under the condition of the centrifugation. Each fraction was subjected to immunoblotting analysis.

Assay for processing activity—Processing activity was assayed essentially as described previously (Hara-Nishimura et al. 1991). A chemically synthesized decapeptide, Ser-Glu-Ser-Glu-Asn-Gly-Leu-Glu-Glu-Thr, was used as the substrate. The peptide sequence was derived from the sequence around the processing site of proglobulin, the proprotein precursor of 11S globulin, a major seed protein of pumpkin (Hayashi et al. 1988b). The reaction mixture contained 4.5 nmol of the decapeptide substrate and the crude extract in 5 μl of 20 mM sodium acetate (pH 5.5), 0.1 M dithiothreitol and 0.1 mM EDTA. The mixture was incubated for 10 to 30 min at 37°C and the products of the reaction were subjected to analytical capillary electrophoresis (model 270A; Applied Biosystems, U.S.A.) at 30°C and 20 kV in 10 mM sodium borate buffer (pH 9.0). Electrophoresis was monitored in terms of absorbance at 200 nm. The vacuolar processing enzyme cleaves only the peptide bond on the C-terminal side of the asparagine residue of the substrate decapeptide to generate an N-terminal pentapeptide P1 and a C-terminal pentapeptide P2. One unit of activity was defined as the amount that liberated 1 μmol of pentapeptide P2 per min under the conditions of the reaction.

Production of antisera against the vacuolar processing enzyme—The vacuolar processing enzyme was purified from castor bean endosperm as described by Hara-Nishimura et al. (1991). Highly purified processing enzyme was injected subcutaneously into a rabbit with complete Freund's adjuvant. After 3 weeks, two booster injections with incomplete adjuvant were given at 7-day intervals. After 1 week, blood was drawn and the antiserum was prepared. Alternatively, a fusion protein, which was consisted of the vacuolar processing enzyme fused to a maltose-binding protein, was expressed in *E. coli* cells. The expressed fusion protein was subjected to SDS-PAGE and after staining protein components with Coomassie Blue R-250, the band corresponding to the protein was cut out from the gel and was used for immunization as described above.

Immunoblotting analysis—Extracts from each tissue were subjected to SDS-PAGE on a 12.5% polyacrylamide gel and the separated proteins on the gels were elec-

trophoretically blotted to GVHP membranes (Millipore, U.S.A.). The immunoblotting reaction was carried out using a specific antibody against the processing enzyme purified from castor bean and alkaline phosphatase conjugated protein A (Cappel, U.S.A.).

Immunocytochemical analysis—Endosperms of castor bean were harvested at the middle stage of seed development and were cut into 1-mm-thick slices with a razor blade. The slices were treated for 2 h with a fixative that contained 4% paraformaldehyde and 1% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4). The sections were dehydrated in a graded dimethylformamide series at −20°C and embedded in LR White (The London Resin Co. Ltd., U.K.). Blocks were polymerized under a UV lamp at −20°C for 24 h. Thin sections were cut on a diamond knife in a Reichert ultramicrotome and mounted on nickel grids. Immunocytochemical procedures for labeling with protein A-gold were essentially the same as those described earlier by Nishimura et al. (1993). Ultrathin sections were incubated at room temperature for 1 h with each solution of an antibody against the purified vacuolar processing enzyme diluted 1 : 2,000 to 10,000, antibody against the above fusion protein expressed in *E. coli* diluted 1 : 200 or antibody against castor bean 11S globulin diluted 1 : 500 to 1,000 in the blocking solution, and then with 25-fold diluted protein A-gold (15 nm; Amersham Japan, Tokyo) at room temperature for 30 min. The sections were examined under an electron microscope at 80 kV (1200EX; JEOL, Japan).

Results

Suborganellar localization of the vacuolar processing enzyme in protein bodies of dry castor bean seeds—Protein bodies found in dry seeds are formed from the vacuoles during seed maturation (Hara-Nishimura et al. 1987). They are membrane-bound organelles in which 11S globulin, a major protein component of castor bean seeds, forms inclusions known as crystalloids (Hara-Nishimura et al. 1982). To characterize the localization of the vacuolar processing enzyme in protein bodies, we isolated protein bodies from dry castor bean seeds and fractionated them into a soluble matrix fraction and the insoluble crystalloid. Ricin was also recovered in the crystalloid fraction under the condition used here.

Immunoblotting analysis using a specific antibody against vacuolar processing enzyme revealed that the enzyme was located in the soluble portion of the protein bodies and was not associated with the crystalloid (Fig. 1). Molecular mass of the enzyme was estimated to be 37 kDa, which coincides with that of the purified vacuolar processing enzyme (Hara-Nishimura et al. 1991), although the migration of the enzyme in the protein body fraction was disturbed by the major seed proteins on SDS-PAGE (Fig. 1). When twice amount of the matrix fraction of protein bodies was applied to SDS-PAGE, another 43-kDa band could be detected on the blot (discussed below). The localization of the vacuolar processing enzyme in the matrix of the protein bodies of dry seeds is supported by our results of immunocytochemical analysis using maturing castor bean seeds (Hara-Nishimura et al. 1993b) and suborganellar fractionation of the vacuoles isolated from maturing pumpkin cotyledons (Hara-Nishimura and Nishimura 1987).

Dense vesicles are involved in the intracellular transport of vacuolar processing enzyme—To characterize intracellular transport of vacuolar processing enzyme, we performed an immunocytochemical analysis of maturing castor bean endosperm using a specific antiserum against vacuolar processing enzyme. The dense vesicles of 300 nm in diameter were labeled with gold particles as well as the vacuolar matrix, an indication that a protein related to vacuolar processing enzyme was localized in the vesicles (Fig. 2A, B, C). These vesicles were different from Golgi

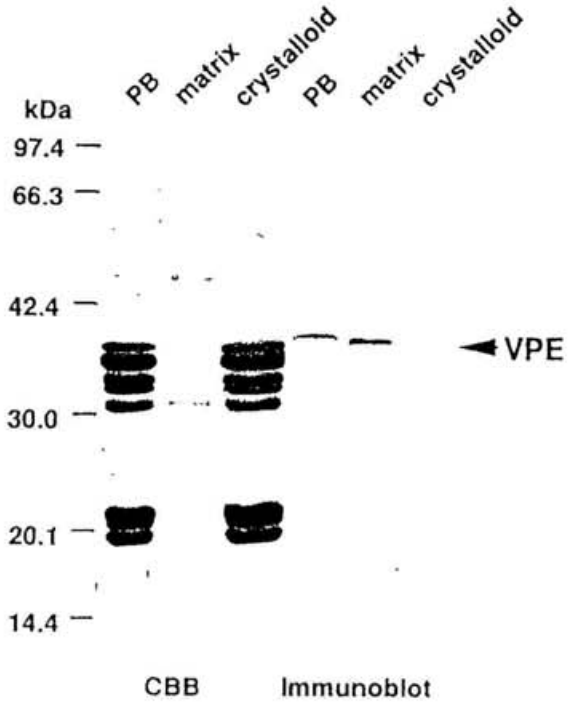

Fig. 1 Suborganellar localization of the vacuolar processing enzyme in protein bodies of endosperm of dry castor bean. Protein bodies (PB) isolated from dry castor bean seeds were separated into a soluble matrix and the insoluble crystalloid. Each fraction was subjected to SDS-PAGE and the proteins were stained with Coomassie Blue (CBB; left). The immunoblotting analysis was performed using a specific antiserum against the vacuolar processing enzyme of castor bean (Immunoblot; right). VPE represent the vacuolar processing enzyme.

vesicles, which were 50 nm in diameter.

Cell fractionation of pulse-chase labeled castor bean endosperm revealed that proglobulin, precursor of 11S globulin, is transported from ER to the vacuoles via dense vesicles (Fukasawa et al. 1988). Immunoelectron microscopy using a specific antibody against 11S globulin showed that gold particles were detected in the dense vesicles of 300 nm in diameter as well as in both vacuolar matrix and the crystalloid (Fig. 2D, E, F). These results suggest that vacuolar processing enzyme accumulates in the dense vesicles of 300 nm in diameter and is then targeted to the vacuoles as 11S globulin does.

Developmental changes in activity of the vacuolar processing enzyme and in total protein content during maturation and germination of castor bean seeds—The activity of the vacuolar processing enzyme was defined as the activity

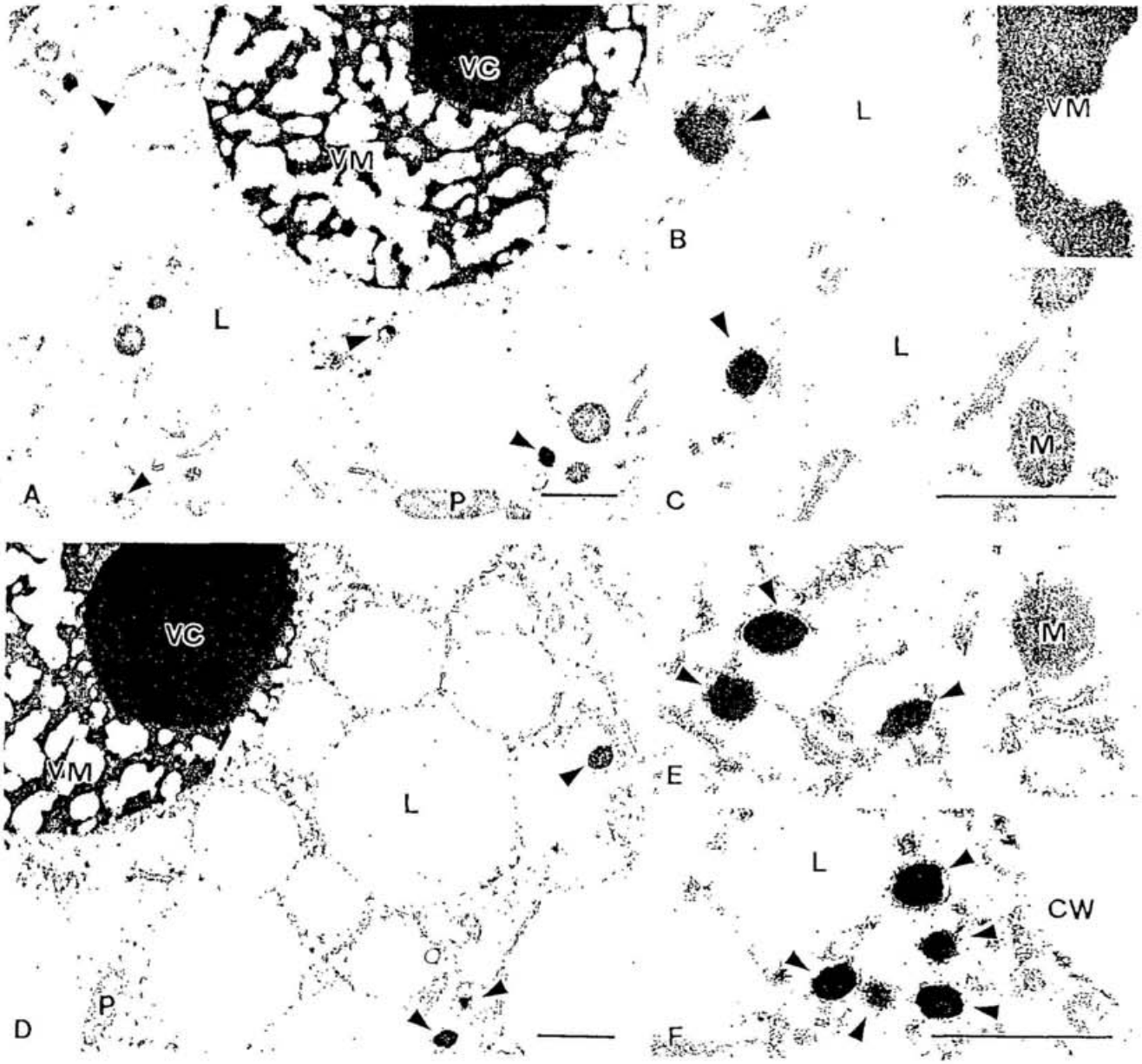

Fig. 2 Dense vesicles involved in the transport of the vacuolar processing enzyme in maturing endosperm cells of castor bean. The labeling of the native vacuolar processing enzyme (A, B, C) and 11S globulin (D, E, F) on a section of a maturing castor bean endosperm is shown. Dense vesicles are indicated by arrowheads. VM, VC, L, M, P and CW represent vacuolar matrix, vacuolar crystalloid, lipid body, mitochondrion, plastid and cell wall, respectively. Bar = 1 μm.

that cleaved the peptide bond on the C-terminal side of the asparagine residue in the synthetic decapeptide, Ser-Glu-Ser-Glu-Asn-Gly-Leu-Glu-Glu-Thr, whose sequence was derived from the sequence around the processing site of proglobulin (Hayashi et al. 1988b). A crude extract from castor bean seeds split the peptide into two pentapeptides, and no further degradation of these two pentapeptides was observed (Hara-Nishimura et al. 1991). This result suggests that only a single activity in the protein body can degrade this decapeptide.

During maturation of castor bean seeds, the accumulation of the vacuolar processing enzyme activity in the endosperm preceded that of total protein (Fig. 3B). The increase in total protein content of the endosperm reflects the biosynthesis of seed proteins. Total protein reached a maximum in dry seeds and the level began to decrease after germination. The processing activity reached a peak at the late stage (III) of the maturation and then decreased during germination of the seeds (Fig. 3B). Four days after the start of germination, endosperm cells started to senesce. After 10 days, the remnants of the endosperm had been completely absorbed by the cotyledons and had disappeared.

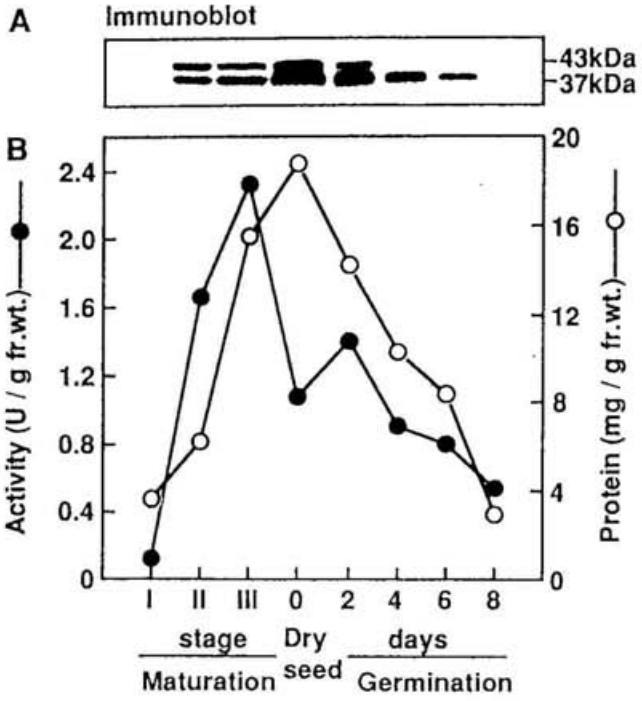

Fig. 3 Developmental changes in the level of the vacuolar processing enzyme and vacuolar processing activity in endosperm tissue during maturation and germination of castor bean seeds. (A) A crude extract prepared at each stage of seed development was subjected to SDS-PAGE that was followed by immunoblotting analysis using an antiserum against the vacuolar processing enzyme. (B) Vacuolar processing enzyme activity was assayed with a synthetic decapeptide as substrate (see text for details). The products of the reaction were analyzed by capillary electrophoresis. The units of enzymatic activity are defined in the text. Closed circles and open circles represent vacuolar processing activity and protein content, respectively. Maturing seeds were harvested at early (I), middle (II) and late (III) stages of seed maturation.

The developmental changes associated with the processing enzyme in the endosperm of castor bean seeds were also examined by immunoblotting analysis using a specific polyclonal antibody against a native vacuolar processing enzyme (Fig. 3A). The amount of the enzyme at the early stage (I) of seed maturation and 8 days after germination was too low to detect by the immunoblotting analysis, as expected from the low enzymatic activity in these tissues. The amount of the enzyme in dry seeds was higher than that expected from the activity. In the case of pumpkin seeds, a maximum activity of proglobulin processing was detected at the late stage of seed maturation and no activity was detected in the dry seeds (Hara-Nishimura et al. 1987). These results suggest that the processing enzyme in the dry seeds may be inactivated, or that the active processing enzyme may be difficult to be extracted from the dry seeds (discussed below).

Vacuolar processing enzyme purified from dry castor

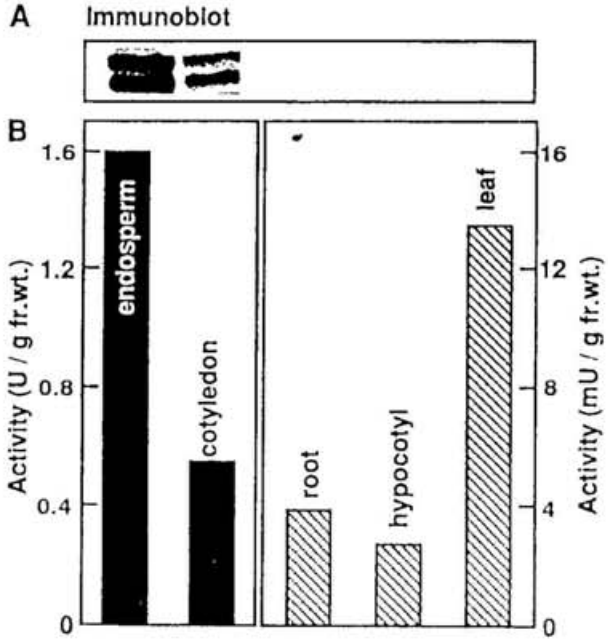

Fig. 4 The vacuolar processing enzyme in various tissues of castor bean. (A) A crude extract of each tissue was subjected to SDS-PAGE and subsequent immunoblotting with an antiserum against the vacuolar processing enzyme. (B) The activity in storage tissues of maturing seeds is shown in U (g fresh weight)$^{-1}$ (left) and the activity in non-storage tissues of seedlings is shown in mU (g fresh weight)$^{-1}$ (right). The assay for the vacuolar processing enzyme was the same as that mentioned in the legend to Figure 3.

bean seeds is 37 kDa (Hara-Nishimura et al. 1991). A 37-kDa polypeptide was detected in the endosperm from maturing and germinating seeds. An immunoreactive 43-kDa polypeptide was also detected in extracts of seeds within 2 days of the start of germination. When we used the antibody raised against the fusion protein of maltose-binding protein and the vacuolar processing enzyme, expressed in *E. coli*, the same immunoblotting profiles were obtained, suggesting that the larger polypeptide of 43 kDa is related to the vacuolar processing enzyme. However, it is still unclear whether the 43-kDa molecule has a processing activity.

Distribution of the vacuolar processing enzyme in various tissues of castor bean—The presence of the processing enzyme in different tissues of castor bean, namely cotyledon, hypocotyl, root and leaf, in addition to endosperm, was examined. The processing activity was calculated on the basis of the fresh weight of each tissue (Fig. 4B). About 30% of the activity in the endosperm was detected in cotyledons from the maturing seeds, and this result was supported by results of immunoblotting analysis (Fig. 4A). In non-storage tissues, such as hypocotyl, root and leaf, we were able to detect 0.2% to 0.8% of the activity found in endosperm (Fig. 4B), although the amounts of the enzyme were too low to detect by immunoblotting analysis (Fig. 4A). When 5-fold amount of the extract was subjected to immunoblotting, the signal was detected on the blot (data not shown). Two immunoreactive polypeptides of 37 kDa and 43 kDa were also detected in cotyledons of maturing seeds, as observed in the case of the endosperm. These results indicate that the vacuolar processing enzyme is present in non-storage tissues, as well as in storage tissues such as the endosperm.

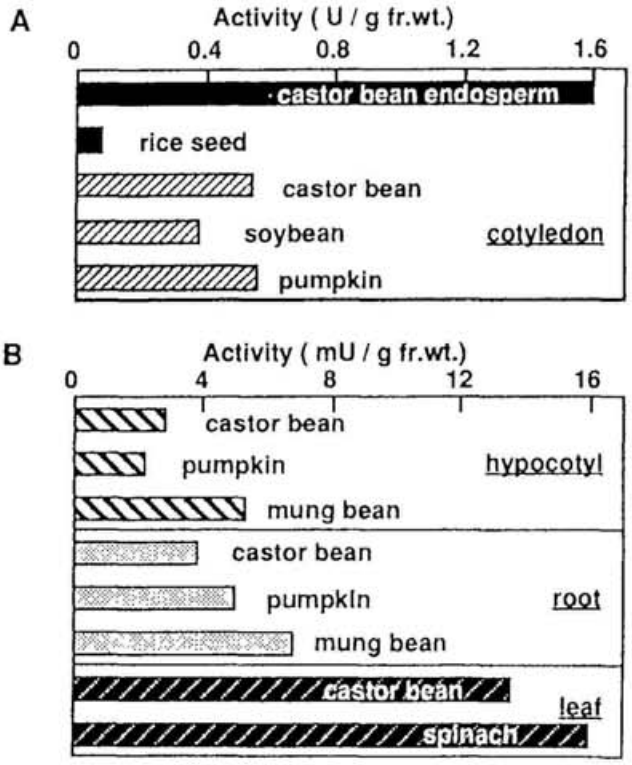

Fig. 5 Distribution of vacuolar processing enzyme in various plants. (A) The activity of the vacuolar processing enzyme in storage tissues of maturing seeds from castor bean, rice, soybean and pumpkin is shown in U (g fresh weight)$^{-1}$. (B) The activity in non-storage tissues of seedlings and mature leaves from various plants is shown in mU (g fresh weight)$^{-1}$. The assay for the vacuolar processing enzyme was the same as that mentioned in the legend to Figure 3.

Distribution of the vacuolar processing enzyme in various plants—We explored the possible presence of processing enzyme activity in various plants. In the cotyledons of maturing pumpkin and soybean seeds, 25% to 32% of the activity found in castor bean endosperm was detected (Fig. 5A). The activity found in monocot rice seeds was less than 5% of that in castor bean endosperm (Fig. 5A). We could also detect the activity in non-storage tissues, which included hypocotyl, root and leaf, although the activity was very low (Fig. 5B). These results suggest that the processing activity is high in storage tissues and is low in non-storage tissues. It should be noted that a vacuolar processing enzyme is involved in the processing of proprotein of vacuolar proteins in non-storage tissues, such as leaf, root and hypocotyl, as well as of seed proteins in storage tissues.

Discussion

Intracellular transport of proprotein precursors of seed proteins was studied by cell fractionation of pulse-chase labeled cotyledons or endosperms at the middle stage of seed maturation. Proprotein precursors of pumpkin 11S globulin (Hara-Nishimura et al. 1985), pumpkin 2S albumin (Hara-Nishimura et al. 1993a) and castor bean 11S globulin (Fukasawa et al. 1988) are transported from rough ER to vacuoles via dense vesicles, whose density is 1.24 g cm^{-3}. Immunoelectron micrograph using antibody against castor bean 11S globulin showed that dense vesicles of about 300 nm in diameter contained 11S globulin-related polypeptide (Fig. 2B). Pro2S albumin of pumpkin was also accumulated in the similar vesicles to those appeared in castor bean (Hara-Nishimura et al. 1993a). Dense vesicles isolated from maturing pumpkin cotyledons contain high levels of a variety of proproteins (Hara-Nishimura et al. 1991). Immunocytochemical analysis in Figure 2 clearly showed that the processing enzyme exists in the vesicles together with proproteins. However, processing of proproteins was not observed in these vesicles (Hara-Nishimura et al. 1985). Thus the processing enzyme must be present in the vesicles as a latent form.

Two immunoreactive polypeptides were detected in the seeds by use of a specific antibody against a native vacuolar processing enzyme. One protein had the same molecular mass of 37 kDa as that of a purified vacuolar processing enzyme and another one had larger molecular mass of 43 kDa than the purified enzyme (Fig. 3A). Both proteins were also detected on an immunoblot with antibodies raised against a fusion protein of a maltose-binding protein and vacuolar processing enzyme expressed in *E. coli* (data not shown). This indicates that the larger polypeptide is related to the processing enzyme. Recently, we succeeded in isolating a cDNA clone for the vacuolar processing enzyme of castor bean (Hara-Nishimura et al. 1993b). The cDNA insert encoded a 55-kDa precursor of the vacuolar processing enzyme. Since the 55-kDa precursor contained a hydrophobic signal sequence at the N-terminus, a putative proprotein precursor of the enzyme is estimated to be about 52 kDa. Further stepwise proteolysis or autolysis of the 52-kDa proprotein must occur to generate an enzyme molecule of 43 kDa and subsequently of 37 kDa.

As discussed above, although various proproteins of seed proteins exist in the dense vesicles together with a precursor of a vacuolar processing enzyme, no processing of the proproteins occurs in the vesicles. Thus a putative 52-kDa precursor is thought to be inactive. The proteolysis or autolysis of the 52-kDa precursor is possibly associated with an activation of the processing activity.

We previously purified a vacuolar processing enzyme from the protein bodies of castor bean endosperm (Hara-Nishimura et al. 1991). The processing activity was assayed with a synthetic decapeptide as substrate, the sequence of which was derived from the sequence around the processing site of the proglobulin molecule (Hayashi et al. 1988b). We reported that the purified processing enzyme can convert pumpkin proproteins that include proglobulin, pro2S albumin and pro51-kDa protein into their respective mature forms (Hara-Nishimura et al. 1991). In a crude extract of protein bodies from castor bean, there is a single proteolytic activity that can cleave the synthetic decapeptide and no further degradation of the produced pentapeptides is observed. Proteolytic activity on casein increased in pumpkin cotyledons during seed germination (Hara and Matsubara 1980). Several proteolytic enzymes are thought to be newly synthesized to degrade storage proteins during seed germination. However, the proteolytic activity on the synthetic decapeptide decreased during seed germination as shown in Figure 3B. This suggests that the decapeptide is resistant to their enzymes. Therefore, we defined the activity of the vacuolar processing enzyme as the activity that cleaves the decapeptide to generate two pentapeptides in the present study.

The accumulation of the activity of the vacuolar processing enzyme in the endosperm preceded that of total proteins during the maturation of castor bean seeds. This result suggests that the processing enzyme plays an important role in the biosynthesis of seed proteins. The developmental change in levels of activity of the processing enzyme in castor bean endosperm is consistent with that in levels of proglobulin-processing activity in pumpkin which converted [^{35}S]proglobulin that was accumulated in the ER prepared from pulse-labeled maturing pumpkin cotyledons into the mature 11S globulin. The proglobulin-processing activity increased during maturation of seeds, but was not detected in dry seeds of pumpkin (Hara-Nishimura and Nishimura 1987). However, we were able to detect the processing of the substrate decapeptide when dry pumpkin seeds were extracted with buffer that contained 0.1 M dithiothreitol and 1 mM EDTA. Depending on the composition of the extraction solution, the processing activity varied. In spite of the accumulation of the enzyme molecules in the dry seeds of castor bean, the activity was lower than that found at the late stage of seed maturation (Fig. 3). The extraction of active processing enzyme from the dry seeds appears to be difficult.

In storage tissues such as cotyledon and endosperm of seed, a single vacuolar processing enzyme can process proprotein precursors of various seed proteins to generate the respective mature polypeptides (Hara-Nishimura et al. 1991). In the case of non-storage tissues, there has been no report on vacuolar processing enzyme. In the present work, we detected the activity of the processing enzyme in non-storage tissues, such as the leaves, hypocotyls and roots of various plants (Figs. 4 and 5). Immunoblotting analysis also showed that the immunoreactive molecule was present in such non-storage tissues of castor bean. These results suggest that a similar processing enzyme to that found in seeds is also present in non-storage tissues.

However, the processing mechanism in vacuoles of non-storage tissues is very obscure. There are two reports on post-translational processing of proteinase inhibitors in non-storage tissues such as tomato leaves (Graham et al. 1985) and stigmas of *Nicotiana alata* (Atkinson et al. 1993). The processing of proprotein precursors of both proteinase inhibitors occurs on the C-terminal side of an asparagine residue as is the case in seed proteins (Hara-Nishimura et al. 1991, 1993a). Thus, a processing enzyme similar to that we found in seeds appears to function in the vacuoles of non-storage tissues.

We observed that the processing activity was high in endosperms and cotyledons and was very low in hypocotyls, roots and leaves. Much larger amounts of protein are synthesized and accumulated in the vacuoles of storage tissues, such as endosperm and cotyledons, than in the vacuoles of leaves and hypocotyls, suggesting that a larger amount of processing enzyme is required in these tissues than in non-storage tissues. As judged from the protein content of the vacuoles of the leaves, hypocotyls and roots of the plants used, the levels of the processing activity seem quite reason-

able. Low levels of the processing activity in these tissues might reflect the low protein content of their vacuoles. It is possible that proteolytic processing is a universal event in the vacuoles of higher plant cells.

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Expression and Activation of the Vacuolar Processing Enzyme in *Saccharomyces cerevisiae*

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Summary

Vacuolar processing enzymes (VPE) are cysteine proteinases responsible for maturation of various vacuolar proteins in plants. A larger precursor to VPE synthesized on rough endoplasmic reticulum is converted to an active enzyme in the vacuoles. We expressed a precursor to castor bean VPE in a *pep4* strain of the yeast *Saccharomyces cerevisiae* to examine the mechanism of activation of VPE. Two VPE proteins with 59 kDa and 46 kDa were detected in the vacuoles of the transformant. They were glycosylated in the yeast cells, although VPE is not glycosylated in plant cells in spite of the presence of two *N*-linked glycosylation sites. During the growth of the transformant, the level of the 59-kDa VPE increased slightly until a rapid decrease occurred after 9-h. By contrast, the 46-kDa VPE appeared simultaneously with the disappearance of the 59-kDa VPE. Vacuolar processing activity increased with the accumulation of the 46-kDa VPE, but not of the 59-kDa VPE. The specific activity of the 46-kDa VPE was a similar level to that of VPE in plant cells. The 46-kDa VPE mediated the conversion of procarboxypeptidase Y to the mature form instead of proteinase A. This indicated that proteinase A responsible for maturation of yeast vacuolar proteins can be replaced functionally by plant VPE. These findings suggest that an inactive VPE precursor synthesized on the endoplasmic reticulum is transported to the vacuoles in the yeast cells and then processed to make an active VPE by self-catalytic proteolysis within the vacuoles.

Introduction

The proprotein precursors of many seed proteins are converted post-translationally to their respective mature forms by the action of vacuolar processing enzymes (VPEs; Hara-Nishimura *et al.*, 1991; Hara-Nishimura *et al.*, 1995; Hara-Nishimura *et al.*, 1993b; Hiraiwa *et al.*, 1993; Kinoshita *et al.*, 1995a; Kinoshita *et al.*, 1995b; Shimada *et al.*, 1994). Several groups have investigated the processing enzymes that convert

proglobulin into 11S globulin in maturing seeds of castor bean (*Ricinus communis*; Hara-Nishimura *et al.*, 1991; Hara-Nishimura *et al.*, 1993b; Hiraiwa *et al.*, 1993), of soybean (*Glycine max*; Muramatsu and Fukazawa, 1993; Scott *et al.*, 1992; Shimada *et al*, 1994) and of pumpkin (*Cucurbita* sp.; Hara-Nishimura and Nishimura, 1987), and that converts proricin to ricin in castor bean (Harley and Lord, 1985). cDNAs for the processing enzymes have been isolated from castor bean (Hara-Nishimura *et al.*, 1993b), soybean (Shimada *et al.*, 1994), *Arabidopsis* (Kinoshita *et al.*, 1995a; Kinoshita *et al.*, 1995b), vetch (Becker *et al.*, 1995), citrus (Alonso and Granell, 1995) and jack bean (Takeda *et al.*, 1994). Molecular characterization of these cDNAs revealed an absence of homology to well-known cysteine proteinases, such as papain (Cohen *et al.*, 1986) and mammalian cathepsin B (Segundo *et al.*, 1985), but 30% to 32% identity at the amino acid level to a putative cysteine protease of *Schistosoma mansoni* (Klinkert *et al.*, 1989). The processing enzymes of plants and the enzyme of *Schistosoma* belong to a novel family of cysteine proteinases.

Recently we isolated three genomic clones for VPE homologues in *Arabidopsis* (Kinoshita *et al.*, 1995a; Kinoshita *et al.*, 1995b). The VPE family can apparently be separated into two subfamilies, one specific to seeds and the other specific to vegetative organs, such as growing cotyledons, leaves and stems. The members of the seed subfamily might function in the protein-storage vacuoles of seeds, while those of the vegetative-organ subfamily might function in the lytic vacuoles of non-storage organs. Thus a similar processing enzyme is widely distributed in plant tissues and plays a crucial role in maturation of a variety of proteins in plant vacuoles.

During seed maturation, the proproteins of seed proteins that have been synthesized on the rough endoplasmic reticulum are transported to the dense vesicles and then into protein-storage vacuoles, where they are processed to the mature forms (Akazawa and Hara-Nishimura, 1985; Fukasawa *et al.*, 1988; Hara-Nishimura *et al.*, 1985; Hara-Nishimura *et al.*, 1993a). Immunocytochemical analysis showed that VPE was selectively localized in the dense vesicles of pumpkin, as well as in the protein-storage vacuoles (Hiraiwa *et al.*, 1993), an indication that VPE is transported to vacuoles via

dense vesicles together with proprotein substrates. However, the endogenous processing of proproteins was not observed in dense vesicles isolated from maturing pumpkin cotyledons. Characterization of molecular structure of the vacuolar processing enzyme is required to elucidate a mechanism of activation of the enzyme.

Previously the precursor to VPE expressed in *Escherichia coli* had no vacuolar processing activity, but a 36-kDa immunopositive protein expressed with a deletion mutant in *E. coli* was active (Hara-Nishimura *et al.*, 1993b). This result showed that the precursor to VPE in the prevacuolar compartment was a latent form and the precursor was converted proteolytically into the active form upon arrival in the protein-storage vacuoles, after fusion of the dense vesicles and the vacuoles (Hara-Nishimura *et al.*, 1995; Hara-Nishimura *et al.*, 1993b). Therefore, the post-translational removal of propeptides from the precursor is required for activation of the VPE. It is well known that proteolytic processing induces a conformational change of many inactive precursors to produce physiologically active proteins (Neurath, 1989). The VPE responsible for conversion of various proproteins plays a crucial role in the biosynthesis of vacuolar components and regulates the biogenesis of protein-storage vacuoles in seeds.

The expression system in *E. coli* was not able to accumulate the VPE because of the toxic effect of activated VPE. We selected the yeast *S. cerevisiae* expression system. Several plant proteins have been expressed in the yeast *S. cerevisiae* and demonstrated to have large capacity to produce a variety of different functional proteins including storage proteins from plants and localized in the vacuole (Matsuoka and Nakamura, 1992; Tague and Chrispeels, 1987; Yarwood *et al.*, 1987). Papain is reported to be accumulated as a glycosylated form of the inactive precursor within in yeast cells defective in the synthesis of vacuolar proteinases, and the complete conversion of the intracellular zymogen into active mature papain could be achieved in vitro (Vernet *et al.*, 1993). Here we report the activation of the VPE expressed in *S. cerevisiae* in the vacuole. The precursor of carboxypeptidase Y (CPY), which is one of the yeast vacuolar proteinases, is known to be processed at peptide bonds on the C-terminal side of asparagine residues into the

mature form (Valls *et al.*, 1987). We used the processing of the precursor of the enzyme as a maker of vacuolar processing activity in vivo.

Results

Expression of castor bean VPE in yeast cells

We constructed the plasmid pYES2-*ppVPE* in which a cDNA encoding a precursor to castor bean VPE was flanked with the *GAL1* promoter and *CYC1* termination sequences of the yeast expression vector, pYES2. The plasmid was introduced into both wild-type (*PEP4*) and proteinase A-deficient (*pep4*) strains of yeast. Immunoblot analysis of these transformants was performed using VPE-specific antibodies, as shown in Figure 1. The *PEP4* transformant grown in SC-glucose medium did not express VPE proteins (Figure 1, lane 1). When the *PEP4* transformant was grown in the presence of galactose, two immunopositive bands of 59 kDa and 46 kDa were detected on the blot of the transformant (Figure 1, lane 2).

To clarify whether or not a 46-kDa protein was a degradation product of a 59-kDa protein generated by proteolytic enzymes in yeast vacuoles, we performed an immunoblot analysis on products from the proteinase A-deficient (*pep4*) transformant. Proteinase A is responsible for maturation and activation of various proteolytic enzymes, such as proteinase B, CPY and proteinase A itself. Thus, the levels of proteolytic activities are extremely reduced in *pep4* cells. No immunopositive bands were found in the *pep4* transformant grown in the SC-glucose medium (Figure 1, lane 3) or the *pep4* cells that were transformed with pYES2 vector and grown in the SC-galactose medium (Figure 1, lane 5). When the *pep4* transformant harboring pYES2-*ppVPE* was grown in the presence of galactose, the transformant exhibited two immunoreactive proteins of 59 kDa and 46 kDa (Figure 1, lane 4), as the *PEP4* transformant did. This suggests that the

46-kDa protein was not an artificial product from the 59-kDa protein by proteolytic enzymes in yeast cells.

Conversion of the 59-kDa VPE to the 46-kDa VPE during the growth of the pep4 *transformant*

Previously, we reported that a proprotein precursor to VPE is synthesized on the rough endoplasmic reticulum (rER) and then converted into a mature form in the protein-storage vacuoles of the seeds of castor bean (Hara-Nishimura *et al.*, 1993b; Hiraiwa *et al.*, 1993). This raises the question of whether or not the 59-kDa protein and the 46-kDa protein correspond to a VPE precursor and a mature VPE, respectively. To answer this question, we examined the conversion of the 59-kDa to the 46-kDa protein during the incubation of the *pep4* transformant in the SC-galactose medium, as shown in Figure 2. The 59-kDa protein could be detected in the transformant cells after 1-h incubation, prior to the detection of the 46-kDa protein after a 3-h incubation. The level of the 59-kDa protein increased to reach the maximum level after a 3-h incubation of the cells, and decreased thereafter. The reduction of the 59-kDa protein was associated with the accumulation of the 46-kDa protein in the cells. This observation indicated that a 59-kDa precursor protein (proVPEsc) was converted to a mature 46-kDa protein (mVPEsc) in the *pep4* transformant cells during growth in the galactose-containing medium, and that proteinase A was not involved in this conversion in the cells.

VPE protein, a non-glycoprotein in plant, was glycosylated in yeast cells

The VPE cDNA that was introduced into the yeast cells encoded a 55-kDa preproprotein precursor with a signal sequence at the N terminus. After co-translational cleavage of the signal sequence, a 53-kDa proprotein precursor to VPE (proVPE) is generated on the rER in the cells of maturing castor bean seed (Hara-Nishimura *et al.*, 1995; Hara-Nishimura *et al.*, 1993b). Thus, the 59-kDa proVPEsc expressed in the transformant

cells was 6-kDa larger than the 53-kDa proVPE in the plant cells. We previously reported that the 53-kDa proVPE of castor bean has two sites of possible *N*-linked glycosylation, but is not glycosylated (Hara-Nishimura *et al.*, 1991; Hara-Nishimura *et al.*, 1993b). It therefore seemed likely that the 53-kDa proVPE was glycosylated in the transformant cells to produce the 59-kDa proVPEsc.

To clarify whether the 59-kDa proVPEsc and the 46-kDa mVPEsc were glycosylated, these proteins were treated with *N*-glycosidase F to remove *N*-linked oligosaccharide from putative glycoproteins (Chu, 1986). Cell extracts were prepared from the *pep4* cells that were transformed with pYES2-*ppVPE* and grown for 1 and 25 h in the galactose-containing medium. These extracts were incubated with *N*-glycosidase F and then subjected to immunoblotting with either VPE-specific antibodies or CPY-specific antibodies. CPY is *N*-glycosylated at four sites of the polypeptide (Valls *et al.*, 1987) and the molecular mass of the oligosaccharides is ~10-kDa (Hasilik and Tanner, 1978). CPY was used as a positive control for the deglycosylation treatment of the cell extracts from the transformant. Figure 3a shows an immunoblot analysis of the CPY with or without the treatment of deglycosylation. After an 18-h incubation of the extracts with *N*-glycosidase F, the 61-kDa CPY (Figure 3a, lane 5, mCPY) was converted into the 51-kDa form that corresponded to the deglycosylated CPY (Figure 3a, lane 6, mCPYΔΨ).

The 59-kDa proVPEsc was detected in the *pep4* transformant after 1-h growth in the galactose-containing medium, while the 46-kDa mVPEsc was detected in the transformant after 25-h growth in the medium (Figure 2). Neither proVPEsc nor mVPEsc was reduced in their molecular masses by a 15-h incubation without *N*-glycosidase F (Figure 3a, lanes 1 and 3). In contrast, the 15-h treatment with *N*-glycosidase F reduced the molecular masses of the 59-kDa proVPEsc and the 46-kDa mVPEsc to 53 kDa and 40 kDa, respectively (Figure 3a, lanes 2 and 4). After 1-h enzyme treatment of the 59-kDa proVPEsc and the 46-kDa mVPEsc, we detected a 56-kDa intermediate and a 43-kDa intermediate, respectively (data not shown). The two stepwise reductions in their molecular masses by 3 kDa might be caused by removal of

two 3-kDa oligosaccharide chains from the glycoproteins. This was supported by the previous report that proVPE contains two possible glycosylation sites (Hara-Nishimura *et al.*, 1993b). These findings suggested that both the 59-kDa proVPEsc and the 46-kDa mVPEsc were *N*-linked glycosylated in the transformant cells.

Figure 3b shows that the molecular mass of the 40-kDa deglycosylated form (lane 2) corresponded with that detected in the endosperm of castor bean (lane 1). Further incubation of the deglycosylated product gave a new band with 37 kDa on an immunoblot (Figure 3b, lane 2). The 37-kDa product also corresponded with the mature VPE from maturing endosperm of castor bean (lane 1). This result suggested that a similar processing of VPE proteins occurred in plants and yeast (discussed below).

Subcellular localization of the VPE proteins in the transformant cells

To examine the localization of proVPEsc and mVPEsc, we performed subcellular fractionation of the *pep4* transformant cells that had been grown in the galactose-containing medium. Three fractions, S100, P13 and P100, were prepared from the *pep4* transformant cells by centrifugation as described by Horazdovsky and Emr (1993). They reported that a marker protein of vacuoles, alkaline phosphatase, was accumulated in the P13 fraction. Figure 4 shows that a marker protein, CPY, was detected in P13 fraction (lane 3), but not in either S100 (lane 2) or P100 fraction (lane 4). Both proVPEsc and mVPEsc were also found in the P13 fraction, but not in the S100 fraction or the P100 fraction, as CPY was (Figure 4, lanes 2 to 4). No immunoreactive products with VPE-specific antibodies were detected in the culture medium of the transformant (data not shown). These findings indicated that both proVPEsc and mVPEsc were localized in the vacuoles of the transformant cells.

Proteinase A may be involved in the maturation of CPY and proCPY is not converted into mature CPY in the *pep4* cells (Jones *et al.*, 1982). However, we found the mature form of CPY in the *pep4* cells transformed with pYES2-*ppVPE* (Figure 3a, lane 5; Figure 4, lanes 1 and 3). This observation suggested that proCPY was converted

into the mature CPY by the action of mVPEsc within the vacuoles of the transformant cells (discussed below).

The 46-kDa VPE exhibited a significant activity of vacuolar processing, but the 59-kDa VPE did not.

To examine whether or not an active VPE molecule was expressed in the transformant, we measured the VPE activity in the cells. The activity was defined as the activity that cleaved the peptide bond on the C-terminal side of the asparagine residue in the synthetic decapeptide, whose sequence was derived from the sequence around the processing site of proglobulin (Hara-Nishimura *et al.*, 1991; Hayashi *et al.*, 1988). No VPE activity was detected in the extracts from the *PEP4* and *pep4* cells that were untransformed and transformed with a vector pYES2 alone (data not shown). The *pep4* transformant with pYES2-*ppVPE* exhibited the VPE activity and the level of the VPE activity increased during growth in the galactose-containing medium, as shown in Figure 5. The increase in the VPE activity was accompanied by the increase of the amount of mVPEsc during the growth in the medium, but not by that of proVPEsc (Figure 2 and Figure 5). These findings suggested that mVPEsc might be an active enzyme and proVPEsc might be an inactive form and that the inactive proVPEsc might be converted to the active mVPEsc in the transformant cells during the growth in the galactose-containing medium.

The results raised the question whether or not the specific activity of mVPEsc was a similar to that of active VPE in plants. To answer the question, we compared the specific activity between the mVPEsc expressed in the transformant and VPE in the protein-storage vacuoles of castor bean. Protein-storage vacuoles (protein bodies) were isolated from dry seeds of castor bean, as described previously and the soluble extract of the protein-storage vacuoles was used as a crude VPE fraction (Hara-Nishimura *et al.*, 1991). The cell extract containing a significant amount of mVPEsc was also prepared from the *pep4* transformant after 25-h in the SC-galactose medium. Each extract exhibiting 0.04 munit of VPE activity was subjected to immunoblot analysis with VPE-

specific antibodies, as shown in Figure 6. The cell extract of the transformant gave a band of the 46-kDa mVPEsc (Figure 6, lane 1), while the extract of the protein-storage vacuoles gave a band of the 37-kDa VPE on the blot (Figure 6, lane 2). The intensity of each signal on the blot was similar. This indicated that the specific activity of mature VPE (mVPEsc) expressed in the transformant cells was the same level as that of mature VPE from plants.

The 46-kDa VPE was involved in the maturation of carboxypeptidase Y in the pep4 *transformant*

We further examined the activity of mVPEsc on proprotein processing in the transformant cells. Maturation and activation of various hydrolytic enzymes, such as proteinase B and CPY, are reported to be regulated by proteinase A (Ammerer *et al.*, 1986; Jones *et al.*, 1982; Woolford *et al.*, 1986). This was supported by the result that maturation of CPY did not occur and proCPY was accumulated in the proteinase A-deficient (*pep4*) strain (Figure 7, lane 2), although mature CPY (mCPY) was accumulated in the *PEP4* strain (Figure 7, lane 1). However, when the proteinase A-deficient (*pep4*) strain was transformed with pYES2-*ppVPE*, mature CPY was accumulated in the *pep4* transformant cells, as shown in Figure 7 (lane 3). Such conversion was not observed in the *pep4* strain transformed with the vector pYES2 alone (Figure 7, lane 4). These findings strongly indicated that the active mVPEsc expressed in the vacuoles of the *pep4* transformant was responsible for the conversion of proCPY into mCPY, instead of proteinase A.

ProCPY is reportedly processed at the C-terminal side of an asparagine residue to generate a mature CPY (Valls *et al.*, 1987), an indication that the asparagine residue at the processing site of proCPY is accessible to VPE. Previously, we reported that VPE recognizes exposed asparagine residues on the molecular surface of proprotein precursors and cleaves the peptide bond on the C-terminal side of each asparagine residue to produce mature protein (Hara-Nishimura *et al.*, 1993a). This evidence suggested that

the mVPEsc expressed in the transformant cells mediated the conversion of proCPY into the mature form in the vacuoles.

Discussion

The VPE precursor is transported to the vacuoles and then post-translationally processed to generate mature form in yeast cells

Previously we reported that the VPE precursors, preproVPEs, are composed of a signal peptide, an N-terminal propeptide, the mature VPE polypeptide and a C-terminal propeptide, as described previously for the VPE of castor bean (Hara-Nishimura *et al.*, 1993b), *Arabidopsis* (Kinoshita *et al.*, 1995a; Kinoshita *et al.*, 1995b) and soybean (Shimada *et al.*, 1994). In maturing seeds, the proVPE synthesized on rER is delivered to the dense vesicles and then to the protein-storage vacuoles, where the proVPE is converted to the mature VPE after cleavage of both N-terminal propeptide and C-terminal propeptide (Hara-Nishimura *et al.*, 1995). The questions are the subcellular localization and the post-translational processing of the VPE proteins, when they were expressed in the yeast cells.

Figure 4 shows that the VPE proteins with 59 kDa and 46 kDa are accumulated in the P13 fraction of the yeast. The P13 fraction contained almost the entire amount of a soluble vacuolar protein, CPY, obtained from whole cell lysates (Figure 4, lower panel). Recently, we found that α-TIP (tonoplast-intrinsic protein) expressed in the same strain of yeast was immunocytochemically localized in the vacuolar membranes of the transformant cells and that the protein was detected in the membranes from P13 fraction of the cells (Inoue *et al.*, 1997). These results indicated that the P13 fraction was regarded as a vacuole-rich fraction and that both 59-kDa and 46-kDa VPE proteins were localized in the vacuoles of the yeast. The 59-kDa VPE corresponds to the VPE precursor and converts to mature 46-kDa VPE, as shown in Figure 2. This strongly

indicates that the post-translational conversion occurs in the vacuoles of the yeast, as in plant cells. However, the proVPE protein could not be detected in plant cells, an indication that the proVPE is converted to the mature form immediately after arriving at the vacuoles in plant cells.

The vacuolar targeting signals of the yeast vacuolar proteins, CPY (Johnson *et al.*, 1987; Valls *et al.*, 1987) and proteinase A (Klionsky *et al.*, 1988), have been localized to the propeptide of the respective precursors. The signal for CPY is identified to be the Gln-Arg-Pro-Leu (QRPL) sequence in the N-terminal region of the propeptide (Valls *et al.*, 1990). The deduced primary sequence of the preproVPEs, however, does not contain the QRPL sequence (Hara-Nishimura *et al.*, 1995), as proteinase A does not. Thus, different receptors have been proposed to recognize different signal sequences for vacuolar targeting (Rothman *et al.*, 1989; Valls *et al.*, 1990).

Self-catalytic conversion of VPE precursor to mature form is associated with activation of VPE

The previous molecular characterization of the VPE shows that the activation of the VPE requires proteolytic removal of the propeptide fragments from the VPE precursor (Hara-Nishimura *et al.*, 1993b; 1995). The present results strongly suggest that the 59-kDa VPE precursor is an inactive form. The level of the VPE activity increased in parallel with the increase of the amount of the 46-kDa VPE, but not the 59-kDa VPE precursor, during the growth of the transformant cells. This suggests that the proteolytic removal of the propeptides from the 59-kDa VPE could elicit a number of changes in the VPE molecule that could result in activation of the cysteine proteinase. The 46-kDa VPE should be characterized as an active site of VPEs that belongs to a novel cysteine proteinase family.

Proteolytic maturation of the VPE occurred in the proteinase A-deficient (*pep4*) transformant cells. In a *pep4* strain, the proteinases in the vacuoles are inactive because proteinase A, which is required for the activation of various vacuolar proteinases, is

deficient (Jones *et al.* 1982). Thus, the conversion of the 59-kDa proVPEsc to the 46-kDa mVPEsc might be mediated self-catalytically. When the mutant VPE in which Cys-83 was substituted with glycine was expressed in yeast cells, the transformant accumulated the 59-kDa proVPEsc, but not the 46-kDa mVPEsc, and showed no VPE activity (unpublished data). The result strongly suggests that self-catalytic autolysis might be responsible for the generation of the mVPEsc. Therefore, it is likely that the proVPE delivered to the protein-storage vacuoles is converted to the mature VPE by self-catalytic mode in the maturing seeds of plants.

The VPE proteins are N*-linked glycosylated in the yeast cells, but not in plant cells*

We previously reported that a 37-kDa VPE purified from castor bean is not a glycoprotein, although the preproVPE sequence contains two possible sites of *N*-glycosylation, Asn-320 and Asn-374, that are included in the mature VPE sequence (Hara-Nishimura *et al.*, 1993b). Thus, these asparagine residues might not be susceptible to the glycosyl transferase that is involved in glycosylation of a precursor to ricin, a major seed protein within the same cells of maturing castor bean. In contrast, the glycosylated proteins, proVPEsc and mVPEsc, were accumulated in the cells of the yeast transformed with pYES2-*ppVPE*, as shown in Figure 3a. They may be glycosylated on both Asn-320 and Asn-374 each with a 3-kDa oligosaccharide. This suggests that the substrate specificity of the glycosyl transferase of the yeast is different from that of the plant enzyme.

A possible manner for self-catalytic proteolytic processing of VPE precursor

The glycosylated 46-kDa VPE exhibited a specific activity similar to the VPE in plants (Figure 6), an indication that glycosylation of the enzyme showed no effect on the proteolytic activity of VPE. Deglycosylation from the 46-kDa VPE gave the 40-kDa VPE and subsequent incubation gave the 37-kDa VPE (Figure 3b). Both 40-kDa and

37-kDa VPEs were also detected in the crude extract freshly prepared from maturing seeds (Figure 3b; Hiraiwa *et al.*, 1993). The result indicates that a similar proteolytic processing of proVPE occurs to generate mature form in yeast and plant cells. The 40-kDa VPE can be regarded as an intermediate and a stepwise proteolytic processing might play a role in self-catalytic maturation of VPE.

Recently, a VPE homologue from vetch was shown to act toward aspartic acid as well as asparagine (Becker *et al.*, 1995). When used a synthetic peptide, Pro-Ser-Leu-Asp-Asp-Glu-Phe-Asp-Leu-Glu-Asp-Asp-Ile-Glu-Asn-Pro-Gln-Gly, as substrate, castor bean VPE favorably cleaved after Asp-Asp sequence (unpublished data). Proteolytic processing of an N-terminal propeptide of jack bean enzyme occurs after Asp-Asp sequence that is conserved among various VPEs (Abe *et al.*, 1993; Takeda *et al.*, 1994). The dipeptide sequence was also conserved around C-terminal processing sites of various VPEs from castor bean (Hara-Nishimura *et al.*, 1993b), jack bean (Takeda *et al.*, 1994), soybean (Shimada *et al.*, 1994), pumpkin (unpublished data) and *Arabidopsis* (Kinoshita *et al.*, 1995a; Kinoshita *et al.*, 1995b). Two Asp-Asp sequences were found at 54th through 57th residues and at 429th and 430th residues of castor bean VPE. It seemed therefore that proVPE might be converted to the 40-kDa intermediate by a self-catalytic removal of N- and C-terminal propeptides at the C-terminal side of the Asp-Asp dipeptides. The substrate specificity toward aspartic acid by VPEs remains to be solved.

We previously proposed that the Asn-374 located in the most hydrophilic region of proVPE sequence was a possible cleavage site to produce mature 37-kDa VPE of castor bean. Abe *et al.* (1993) reported that the jack bean enzyme could not cleave a peptide bond on C-terminal side of *N*-glycosylated asparagine residues. Thus, if Asn-374 is glycosylated in the yeast, the residue could not be involved in self-catalytic processing of VPE protein. The 37-kDa VPE was detected only in the in vitro reactions after deglycosylation of the 46-kDa VPE (Figure 3b). Thus, it is likely that deglycosylation might make the Asn-374 susceptible to active VPE itself. The 40-kDa VPE might be converted to the 37-kDa VPE of the final form by a self-catalytic cleavage at Asn-374.

Asn-374 of castor bean VPE is not conserved among various VPE homologues (Hara-Nishimura *et al.*, 1995). The molecular masses of VPE homologues are generally in the range 33-39 kDa (Abe *et al.*, 1993; Becker *et al.*, 1995; Hara-Nishimura *et al.*, 1991; Shimada *et al.*, 1994; Kembhavi *et al.*, 1993). Thus, we could not exclude the possibility that final processing site for removal of C-terminal fragment was varied among VPE homologues.

Plant VPE take over the function of proteinase A responsible for maturation of yeast vacuolar proteins

The proCPY is known to be processed to form mCPY on the C-terminal side of Asn-111 (Valls *et al.*, 1987). It was reported that the *pep4* strain accumulates proCPY, but not mCPY (Jones *et al.*, 1982). However, the *pep4* transformant cells expressing VPE proteins accumulated mCPY, but not proCPY, as shown in Figure 7. Previously we proposed that the VPE recognizes exposed asparagine residues on the molecular surface of proprotein precursors to various vacuolar proteins in plants (Hara-Nishimura *et al.*, 1991). This suggests that the expressed and activated VPE mediated the maturation of CPY instead of proteinase A. The present study suggests that the seed-type VPE plays a role in maturation of vacuolar proteins in the lytic vacuoles of the yeast cells.

Experimental procedures

Yeast strains, culture conditions and transformation

Yeast *PEP4* strains (YW23-5A; *MAT*a *leu2 ura3-52*) and *pep4* strains (YW7-6D; *MAT*a *leu2 ura3-52 pep4-3*) of *Saccharomyces cerevisiae* were donated by Dr. Y. Wada (University of Tokyo) and used as hosts for transformation. Synthetic medium [SC medium; 0.17% yeast nitrogen base without amino acids (Difco, Detroit, MI, USA)]

containing casamino acid (2%), adenine sulfate (20 mg/l), tryptophan (20 mg/l), leucine (30 mg/l) and histidine (20 mg/l) was used. Cells were grown to stationary phase in the above media supplemented with 2% raffinose (SC-raffinose medium) to enhance induction of expression. The cells were then grown in media supplemented with 2% glucose (SC-glucose medium) or 5% galactose (SC-galactose medium) and harvested for an immunoblot analysis. These cells were transformed by the lithium method with modifications (Ito *et al.*, 1983; Schiestel and Gietz, 1989). Transformant cells were selected for uracil prototrophy.

Construction of plasmids

The entire reading frame of the cDNA insert of precursor (*ppVPE*) of castor bean VPE (Hara-Nishimura *et al.*, 1991) was amplified by PCR using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The PCR primers were designed to contain the *Pvu*II site at the 5'-terminus and *Xho*I site at the 3'-terminus, respectively, for a directional cloning. The amplified 1494-bp fragment for ppVPE was ligated into a *Pvu*II-*Xho*I digested expression vector of pYES2 (Invitrogen, USA) to generate a construct containing the cDNA flanked with the yeast *GAL1* promoter and *CYC1* termination sequences of the plasmids. The nucleotide sequence of the cDNA with pYES2 was determined with an automatic DNA sequencer (model 373A; Perkin Elmer/Applied Biosystems, Foster City, CA, USA) to confirm no mismatches to the sequence of the original cDNA.

Preparation of yeast cell lysates and immunoblot analysis

Whole cell lysates were prepared from either SC-glucose or SC-galactose-grown transformant cells, as described previously (Wada *et al.*, 1992). Cells were collected by centrifugation, sheared mechanically with glass beads (Sigma, St. Louis, MO, USA) and

lysed in SDS sample buffer or 20 mM Tris-HCl (pH 7.5) that contained 1 mM phenylmethanesulfonyl fluoride (PMSF).

Aliquots of the lysates were subjected to SDS-PAGE and subsequent immunoblot analysis. Immunoblot analysis was performed essentially as described previously (Inoue *et al.*, 1995). The separated proteins on SDS-PAGE gels were transferred electrophoretically to a GVHP membrane (Nihon Millipore, Tokyo, Japan). The membrane blot was incubated for 1 h with either VPE-specific antibodies (diluted 1000-fold; against a fusion protein composed of a maltose-binding protein and a vacuolar processing enzyme from castor bean that had been expressed in *E. coli*.) or CPY-specific antibodies (diluted 2000-fold; donated by Dr. Y. Wada in University of Tokyo). Horseradish peroxidase-conjugated antibodies raised in goat against rabbit IgG (Cappel, West Chester, PA, USA) were diluted 10000-fold. Immunodetection was performed with an ECL kit (Amersham Japan, Tokyo) in accordance with the manufacturer's directions.

Deglycosylation treatment with N-*glycosidase F*

Total proteins were extracted in 20 mM Tris-HCl (pH 7.5) with 1 mM PMSF from the transformant cells (*pep4* strains; YW7-6D). To remove oligosaccharides, the proteins (50 μg) were treated with 1 unit of *N*-glycosidase F (Amersham Japan) at 37 °C for 15 h. The deglycosylated proteins were subjected to SDS-PAGE on an 8-12.5% acrylamide gel in the presence of 2-mercaptoethanol and then to immunoblot analysis with either VPE-specific antibodies or CPY-specific antibodies.

Subcellular fra*ctionation of the transformant cells*

The transformant cells (*pep4* strains; YW7-6D) were grown in 20 ml of SC-glucose medium to 0.4 absorbance unit at 600 nm and were then grown in SC-galactose at 30 °C for 12 h. The cells collected by centrifugation were resuspended in 0.1 M Tris-SO_4 (pH

9.5) with 20 mM 2-mercaptoethanol and then incubated for 20 min at 30 °C. The mixture was further incubated in YP medium (1% yeast extract and 2% polypeptone) with 1.3 M sorbitol and 62.5 units of Zymolyase (Seikagaku Corporation, Tokyo, Japan) for 20 min at 30 °C. The spheroplasts collected were suspended in 0.1 M Tris-HCl (pH 7.5) with 1.2 M sorbitol, 10 mM EDTA and 1 mM PMSF.

For subcellular fractionation, the spheroplasts were burst in 0.1 M Tris-HCl (pH 7.5) containing 0.25 M sucrose, 10 mM EDTA, 1.25 mg BSA and 1 mM PMSF and the lysate was subjected to differential centrifugations as described previously (Horazdovsky and Emr, 1993; Nishikawa and Nakano, 1991). The lysates were centrifuged at 13 000 ***g*** for 15 min to generate supernatant (S13) and pellet (P13) fractions. The S13 fraction was further centrifuged at 100 000 ***g*** for 30 min, yielding supernatant (S100) and pellet (P100) fractions. The P13 fraction was regarded as a vacuole-rich fraction. Proteins in each of P13, S100 and P100 fractions were precipitated in 10% trichloroacetic acid and then resuspended with SDS sample buffer. These preparations were subjected to SDS-PAGE and subsequent immunoblot analysis with either VPE-specific antibodies or CPY-specific antibodies.

Isolation and suborganellar fractionation of protein bodies from dry seeds of castor bean

Protein bodies were prepared from dry seeds of castor bean by the glycerol-isolation method, as described previously (Hara-Nishimura *et al.*, 1982; Hiraiwa *et al.*, 1993). Both light-microscopic examination and assays of marker enzymes indicated that the isolated protein bodies were not contaminated by other cell organelles or cytoplasmic components. After lysis of the protein bodies by addition of a hypotonic buffer solution of 10 mM Tris-HCl (pH 7.5), the homogenate was centrifuged at 100 000 ***g*** for 20 min at 4 °C, to remove an insoluble crystalloids that were composed of the major seed proteins, 11S globulin. The supernatant fraction containing 0.04 munits of VPE activity was subjected to SDS-PAGE and subsequent immunoblot analysis with VPE-specific antibodies.

For preparation of a crude extract from castor bean endosperm, 1 g fresh weight of endosperm was homogenized with 2 ml of 10 mM Tris-HCl (pH 7.5) on ice and was centrifuged twice at 20 000 ***g*** for 15 min at 4 °C. The supernatant solution was used as a crude extract from castor bean as described before (Hiraiwa *et al.*, 1993).

Assay for vacuolar processing activity

Activity of vacuolar processing enzyme was assayed essentially as described previously (Hara-Nishimura *et al.*, 1991). A chemically synthesized decapeptide, Ser-Glu-Ser-Glu-Asn-Gly-Leu-Glu-Glu-Thr, was used as the substrate. The peptide sequence was derived from the sequence around the processing site of proglobulin, the proprotein precursor of 11S globulin, a major seed protein of pumpkin (Hayashi *et al.*, 1988). The reaction mixture contained 4.5 nmol of the decapeptide substrate and the crude extract in 5 μl of 20 mM sodium acetate (pH 5.5), 0.1 M dithiothreitol and 0.1 mM EDTA. The mixture was incubated for 3 h at 37 °C and the products of the reaction were subjected to analytical capillary electrophoresis (model 270A; Perkin Elmer/Applied Biosystems, Foster City, CA, USA) at 30 °C and 20 kV in 10 mM sodium borate buffer (pH 9.0). Electrophoresis was monitored in terms of absorbance at 200 nm. The vacuolar processing enzyme cleaves only the peptide bond on the C-terminal side of the asparagine residue of the substrate decapeptide to generate an N-terminal pentapeptide P1 and a C-terminal pentapeptide P2. One unit of activity was defined as the amount that liberated 1 mmol of pentapeptide P2 per min under these conditions.

In vivo processing of proCPY into mature CPY in proteinase A-deficient strain transformed with pYES2-ppVPE

Proteinase A-deficient strain (*pep4*; YW7-6D) was transformed with pYES2-*ppVPE* and was then grown in the SC- galactose medium for 15 h. The *pep4* strain was also transformed with pYES2 and was then grown in the SD-galactose medium for 15 h.

(lane 3) or with pYES2 (lane 4). The cells collected were subjected to immunoblot analysis with CPY-specific antibodies.

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Figure legends

Figure 1. *PEP4*-independent expression and accumulation of two molecular forms of castor bean VPE in the yeast cells transformed with pYES2-*ppVPE*.
Both wild-type strain (*PEP4*; lanes 1 and 2) and proteinase A-deficient strain (*pep4*; lanes 3 and 4) of *Saccharomyces cerevisiae* were transformed with pYES2-*ppVPE*. These transformants were grown to stationary phase in the SC-raffinose medium, before they were grown in the SC-glucose (lanes 1 and 3) or the SC-galactose (lanes 2 and 4) for 8 h. The same number (2×10^7) of the cells of each transformant was subjected to SDS-PAGE and subsequent immunoblot analysis with VPE-specific antibodies. The yeast *pep4* strain transformed with pYES2 was also grown in the SC-galactose medium for 8 h and then harvested for immunoblot analysis (lane 5). Two immunopositive bands of 59 and 46 kDa were found on the blots. The molecular mass of each marker protein is given on the right in kDa.

Figure 2. Conversion of a 59-kDa VPE precursor to a 46-kDa VPE in the *pep4* transformant during incubation in the SC-galactose medium.
The *pep4* cells transformed with pYES2-*ppVPE* were grown in the SC-galactose medium for 1 to 25 h. The cells were harvested at the time indicated. Total proteins (10 μg) of the cells were subjected to SDS-PAGE and subsequent immunoblot analysis with VPE-specific antibodies. proVPEsc and mVPEsc indicate the 59-kDa VPE precursor and the 46-kDa mature VPE, respectively. The molecular mass of each marker protein is given on the right in kDa.

Figure 3. Both the 59-kDa proVPEsc and 46-kDa mVPEsc were glycosylated in the transformant cells.
(a) The *pep4* cells transformed with pYES2-*ppVPE* were grown in the SC-galactose medium for 1 h (lanes 1 and 2) and 25 h (lanes 3 to 6). Each cell lysate was incubated in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of *N*-glycosidase F at 37 °C

for 15 h. The reaction products were subjected to SDS-PAGE and subsequent immunoblot analysis with either VPE-specific antibodies (anti-VPE; lanes 1 to 4) or CPY-specific antibodies (anti-CPY; lanes 5 and 6). mCPY and mCPY$\Delta\Psi$ indicate mature CPY and deglycosylated mature CPY, respectively. (b) A crude extract from maturing endosperm of castor bean seeds (lane 1) and the deglycosylated mature VPE that was further incubated (lane 2) were subjected to immunoblot analysis as described above. The molecular mass of each marker protein is given on the right in kDa.

Figure 4. Both the 59-kDa proVPEsc and 46-kDa mVPEsc were localized in the vacuoles of the transformant cells.

The *pep4* cells transformed with pYES2-*ppVPE* were grown in the SC-galactose medium for 15 h. Spheroplasts from the cells were subjected to differential centrifugations for subcellular fractionation. Total cells (lane 1), S100 fraction (lane 2), P13 fraction (lane 3) and P100 fraction (lane 4) were subjected to SDS-PAGE and subsequent immunoblot analysis with either VPE-specific antibodies (anti-VPE; upper panel) or CPY-specific antibodies (anti-CPY; lower panel). mCPY indicates mature CPY.

Figure 5. Increase in the level of the vacuolar processing enzyme activity in the transformant cells during incubation in the SC-galactose medium.

The *pep4* cells transformed with pYES2-*ppVPE* were grown in the SC- galactose medium for 1 to 25 h. Vacuolar processing enzyme activity was assayed with a synthetic decapeptide as substrate (see text for details). The products of the reaction were analyzed by capillary electrophoresis. The units of enzymatic activity are defined in the text.

Figure 6. Comparison of the specific activity of VPE in the transformant cells with that of VPE from castor bean endosperm.

VPEs were prepared either from the *pep4* cells transformed with pYES2-*ppVPE* or from the protein bodies isolated from dry seeds of castor bean. The cell lysate of the transformant with 0.04 munit of VPE activity (lane 1) and the soluble fraction of the protein bodies with 0.04 munit of VPE activity (lane 2) were subjected to SDS-PAGE and subsequent immunoblot analysis with VPE-specific antibodies.

Figure 7. In vivo processing of proCPY to make mature CPY in proteinase A-deficient strain transformed with pYES2-*ppVPE*.

Non-transformants of wild-type (*PEP4*) strain (lane 1) and proteinase A-deficient (*pep4*) strain (lane 2) and the *pep4 transformants* with pYES2-*ppVPE* (lane 3) and with pYES2 (lane 4) were subjected to SDS-PAGE and subsequent immunoblot analysis with CPY-specific antibodies. proCPY and mCPY indicate CPY precursor and mature CPY.

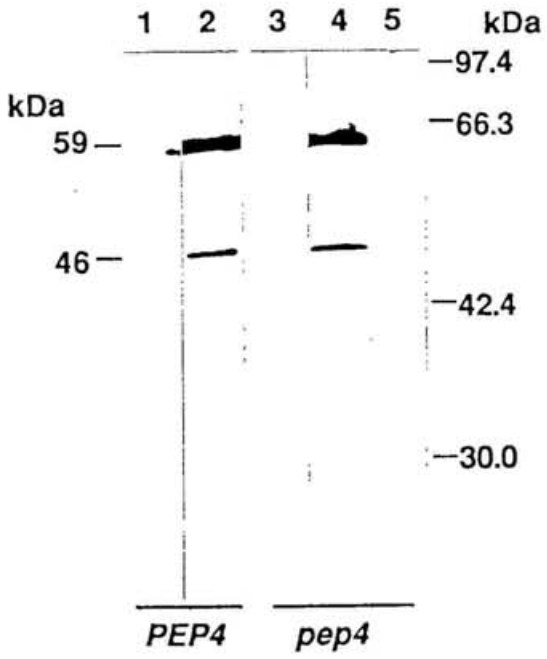
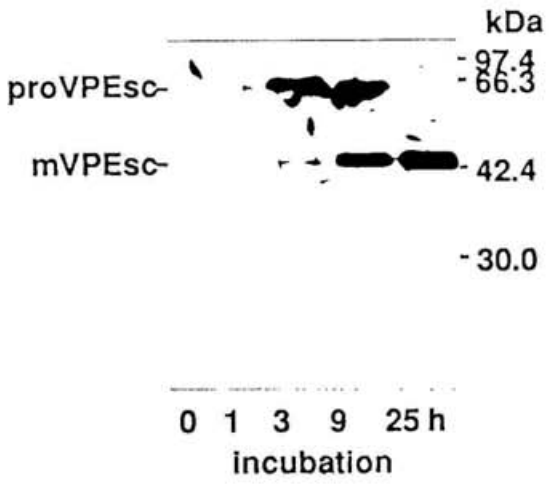


Fig. 1. Hiraiwa *et al.*



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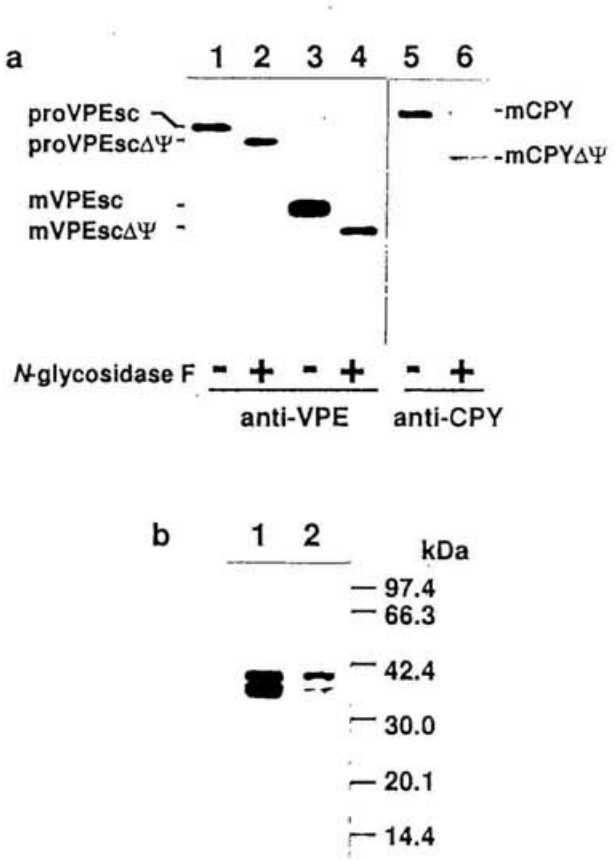

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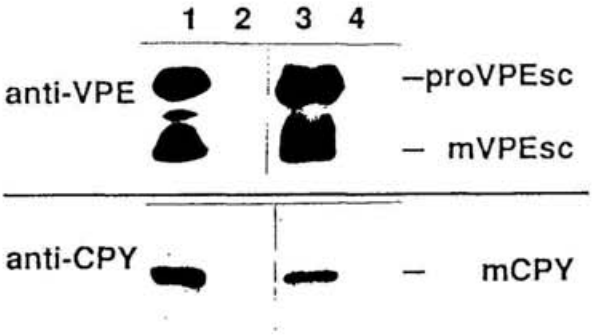

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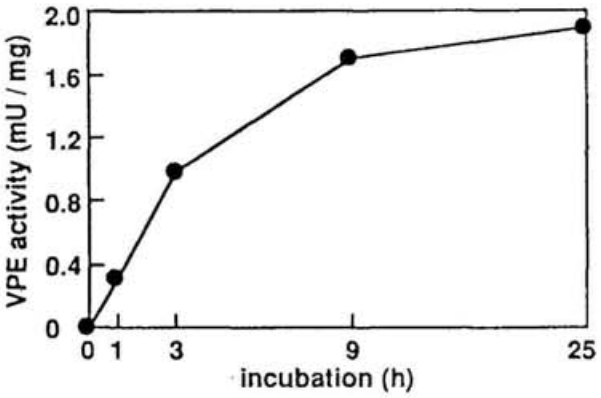

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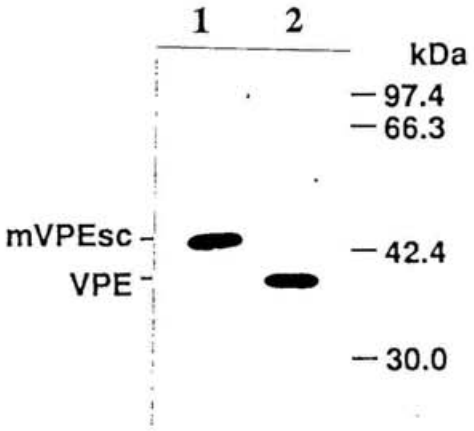

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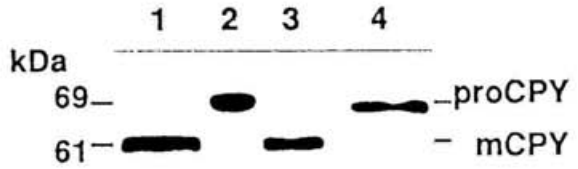

Fig. 7. Hiraiwa *et al.*

An Aspartic Proteinase is Involved in Breakdown of Propeptides of Storage Proteins in Protein-Storage Vacuoles of Plants

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Abbreviations: ER, endoplasmic reticulum; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; RCA, *Ricinus communis* agglutinin; TCA, trichloroacetic acid.

Enzymes. Aspartic proteinase (EC 3.4.23); Vacuolar processing enzyme / Asparaginyl endopeptidase (EC 3.4.22.34)

SUMMARY

To understand the mechanism of the maturation of various proteins in protein-storage vacuoles, we purified a 48-kDa aspartic proteinase composed of a 32-kDa and a 16-kDa subunits from castor bean. Immunocytochemical and cell fractionation analyses of the endosperm of maturing castor bean seed showed that the aspartic proteinase was localized in the matrix of the protein-storage vacuoles, where a variety of seed storage proteins were also present. The amount of the aspartic proteinase increased at mid-maturation stage of the seeds prior to the accumulation of the storage proteins. To determine how the aspartic proteinase is responsible for maturation of seed proteins in concert with the vacuolar processing enzyme, we prepared [^{35}S]proproteins of seed proteins from the endoplasmic reticulum fraction of pulse-labeled maturing endosperm and used the authentic proproteins as substrates for *in vitro* processing experiments. The purified aspartic proteinase was unable to convert any of three endosperm proproteins, pro2S albumin, proglobulin and proricin, into their mature sizes, while the purified vacuolar processing enzyme could convert all three proproteins. We further examined the activity of aspartic proteinase on the cleavage of an internal propeptide of *Arabidopsis* pro2S albumin, that was well known to be post-translationally removed. The aspartic proteinase cleaved the propeptide at three sites under the acidic condition. These results suggest that aspartic proteinase cannot directly convert pro2S albumin into the mature form, but it may play a role in trimming the C-terminal propeptides from the subunits that are produced by the action of the vacuolar processing enzyme.

Keywords: aspartic proteinase; proprotein processing; storage proteins; vacuolar processing enzyme; protein-storage vacuole;

INTRODUCTION

Proprotein precursors of various seed proteins, such as 11S globulin, 2S albumin and lectins, are processed post-translationally to yield the mature proteins [1]. Most of the processing sites are on the C-terminal side of an asparagine residue [2]. We succeeded previously in purifying a vacuolar processing enzyme, a novel cysteine proteinase, from castor bean (*Ricinus communis*) that specifically cleaves a peptide bond on the C-terminal side of an asparagine residue of different proproteins to generate the mature size proteins [2-4]. Some seed proteins, however, contain an internal propeptide and/or a C-terminal propeptide that must be removed [5-7]. Thus, proteolytic cleavages at amino acid residues other than asparagine residues must also be necessary to remove these propeptides. The detailed mechanism for the post-translational processing of vacuolar proteins is still obscure.

2S albumin is one of the best characterized proteins that are localized in protein-storage vacuoles [5]. We demonstrated that the vacuolar processing enzyme cleaves a peptide bond on the C-terminal side of two asparagine residues of proprotein precursor of 2S albumin (pro2S albumin) in pumpkin to generate the mature size protein [3, 8]. The two asparagine residues are conserved in pro2S albumins of various plants [8]. However, pro2S albumin in Brazil nut lacks the second asparagine residue and the intermediate precursor accumulates in the maturing seed for more than 2 months before it is further processed to the mature form [9]. This result suggested that a proteolytic enzyme other than the vacuolar processing enzyme might be involved in the final processing of the intermediate precursor to produce the mature 2S albumin. It remains to be determined when and how the subsequent cleavage occurs in the protein-storage vacuoles.

Pro2S albumins in *Arabidopsis* [5], *Brassica* [10, 11] , castor bean [6, 7] and Brazil nut [12, 13] contain an internal propeptide between the two subunits of the mature protein. The propeptide must be proteolytically removed during seed maturation. Recently, D'Hondt *et al.* [14] reported that an aspartic proteinase from *Brassica napus* cleaves a

synthetic peptide containing the internal propeptide of pro2S albumin-2 of *Arabidopsis*. This suggested that a coordination of the aspartic proteinase and the vacuolar processing enzyme might mediate post-translational processing of pro2S albumin. Runeberg-Roos *et al.* showed that the barley aspartic proteinase is localized in vacuoles of leaf and root cells [15, 16]. It needs to be determined whether an aspartic proteinase is also localized in protein-storage vacuoles of maturing seeds.

Aspartic proteinases have been found in a variety of plant seeds [17]. A precursor to the barley aspartic proteinase [18] is similar to mammalian and yeast aspartic proteinases [19], but a 104-residue insertion is found at the same position as intron 7 in mammalian aspartic proteinases [18]. The insertion is replaced by Loop 6 in the lysosomal aspartic proteinase, cathepsin D [20]. The precursor to the plant enzyme is post-translationally cleaved in the inserted region to make a two-chain enzyme [18, 21]. Runeberg-Roos *et al.* reported that an aspartic proteinase participates in processing the C terminus of barley lectin [15]. The involvement of aspartic proteinases in the maturation of storage proteins, including 2S albumins, needs to be examined.

This study was designed to determine how the aspartic proteinase is involved in the post-translational processing in concert with the vacuolar processing enzyme. We purified both an aspartic proteinase and a vacuolar processing enzyme from protein-storage vacuoles of castor bean and prepared authentic proprotein precursors from the endoplasmic reticulum (ER) and transport vesicle fractions of maturing seeds as substrates for an *in vitro* processing assay to understand the mechanism of the maturation of various proteins in plant vacuoles.

MATERIALS AND METHODS

Materials. Dry seeds of castor bean (*Ricinus communis*) were directly used for isolation of protein-storage vacuoles, and the maturing endosperm was pulse-labeled to

prepare [^{35}S]-labeled proprotein precursors of the seed proteins, as described below. To obtain maturing seeds, castor bean was grown in a greenhouse. Maturing endosperm tissues were harvested at the early, middle and late stages of seed maturation (stages I, II and III) as described previously [22]. These stages corresponded to 15 to 20 days, 25 to 30 days, and 35 to 40 days after anthesis, respectively [22]. To obtain seedlings, castor bean seeds were soaked in water overnight, planted at 25 °C in the dark and allowed to germinate and grow in coarse vermiculite for up to 8 days. Endosperm tissues were harvested from seedlings of the desired ages.

Pumpkin (*Cucurbita* sp. cv, Kurokawa Amakuri Nankin) seeds were purchased from Aisan Shubyo Seed Co. (Nagoya, Japan). The seeds were planted in the field during the summer. The cotyledonous tissues of the maturing seeds were freshly harvested 25 to 30 days after anthesis and were used for the following experiment.

Isolation and suborganellar fractionation of protein-storage vacuoles. Protein-storage vacuoles (protein bodies) were prepared from dry seeds of castor bean by the glycerol-isolation method, as described previously [23, 24]. Both light-microscopic examination and assays of marker enzymes indicated that the isolated protein-storage vacuoles were not contaminated by other cell organelles or cytoplasmic components. After lysis of the protein-storage vacuoles by addition of a hypotonic buffer solution of 10 mM Tris/HCl pH 7.5, the homogenate was separated into a soluble matrix and an insoluble crystalloid by centrifugation at 100000 g for 20 min at 4 °C. The crystalloid was composed of the major seed proteins. The matrix fraction was used as a starting material for purification of aspartic proteinase.

Purification of the aspartic proteinase. The soluble matrix fraction of protein-storage vacuoles was suspended in a buffer of 50 mM sodium acetate pH 3.5 and 0.2 M NaCl and was sequentially subjected to centrifugation to remove insoluble proteins. The supernatant solution was applied to a column packed with 2 ml of pepstatin A agarose (Sigma, St. Louis, MO, USA). The pepstatin A agarose was washed with 4 ml of 50 mM

sodium acetate pH 3.5 and 0.2 M NaCl and then with 10 ml of 50 mM Tris/HCl pH 7.5 and 0.2 M NaCl. Elution of the aspartic proteinase was performed with 50 mM sodium bicarbonate pH 10.0 and 0.2 M NaCl. The affinity chromatography with pepstatin A agarose was repeated twice to obtain a pure aspartic proteinase.

N-terminal amino acid sequence of the aspartic proteinase. The purified aspartic proteinase was subjected to SDS-PAGE with 2-mercaptoethanol on a 15% acrylamide gel and the separated subunits of the enzyme were electrophoretically transferred to a ProBlott^{TM} membrane (Perkin Elmer/Applied Biosystems, Foster City, CA, USA). The subunits with molecular masses of 32, 29 and 16 kDa were subjected to automatic Edman degradation on a protein sequencer (model 476A, Perkin Elmer/Applied Biosystems, Foster City, CA, USA).

Isolation and nucleotide sequence of a cDNA for an aspartic proteinase. A degenerate oligonucleotide, 5'-GATACTGGTTC(GATC)TC(GATC)AA(CT)CTITGGGTICC-3', was designed on the basis of the conserved sequence of an active site of aspartic proteinases (DTGSSNLWVP) and synthesized on a DNA synthesizer (Pharmacia LKB Biotechnology, Sweden). A cDNA library was constructed with a vector (pTTQ18) primer and the poly(A)$^{+}$RNA from maturing pumpkin cotyledons as previously described by Mori *et al.* [25]. *Escherichia coli* cells (Epicuriancoli SCS1 cells that originated from DH1 cells; Stratagene, USA) were transformed with the cDNA and were screened by a colony hybridization method using the oligonucleotide as probe to obtain a cDNA clone for pumpkin aspartic proteinase.

Deletion mutants of both sense and antisense strands of the cDNA insert were constructed. Nucleotide sequences were determined by the dideoxy chain-termination method with Sequenase Version 2.0 (United States Biochemical Corporation, USA) according to the manufacturer's directions.

Production of specific antibodies against the purified aspartic proteinase. The purified aspartic proteinase was injected subcutaneously into a rabbit

with complete Freund's adjuvant. After 3 weeks, two booster injections with incomplete adjuvant were given at 7-day intervals. After 1 week, blood was drawn and the antibodies were prepared. The specificity of the antibodies to the aspartic proteinase was confirmed by immunoblots of the crude extracts of the maturing endosperm (data not shown) and the isolated protein-storage vacuoles (Fig. 2).

Immunoblot analysis. Sample extracts were subjected to SDS-PAGE on a 15% acrylamide gel in the absence of 2-mercaptoethanol and the separated proteins on the gels were electrophoretically transferred to a PVDF (polyvinylidene difluoride) membrane (0.22 μm; Nihon Millipore Ltd., Tokyo, Japan). The membrane blot was incubated in Tris-buffered saline (50 mM Tris/HCl pH 7.5 and 0.15 M NaCl), 0.05% (v/v) Tween 20 and 3% (w/v) skim milk and subsequently incubated in the same solution with antibodies against the aspartic proteinase (diluted 2000-fold) for 1 h. Alkaline phosphatase-conjugated goat antibodies against rabbit IgG (diluted 2000-fold; Cappel, France) were used as second antibodies [26]. The levels of the signal on the blot were determined with a densitometer (AE-6920-M, ATTO Corporation, Tokyo, Japan).

Immunocytochemical analysis. Endosperms of castor bean were harvested at the middle stage of seed development and were cut into 1-mm-thick slices with a razor blade. The slices were treated for 2 hr with a fixative that contained 4% paraformaldehyde and 1% glutaraldehyde in 0.05 M cacodylate buffer pH 7.4. The sections were dehydrated in a graded ethanol series at -20 °C and embedded in LR White (The London Resin Co. Ltd., UK). Thin sections were cut with a diamond knife in an ultramicrotome (Pharmacia LKB Biotechnology, Sweden) and mounted on nickel grids. Immunocytochemical procedures for labeling with protein A-gold were essentially the same as those described earlier by Nishimura *et al.* [27]. Ultrathin sections were incubated at room temperature for 1 hr with either antibodies against the aspartic proteinase (diluted 2000-fold) or antibodies against castor bean 11S globulin, a major storage protein (diluted 2000-fold) in the blocking solution, and then with 25-fold diluted protein A-gold (15 nm; Amersham Japan, Tokyo) at

room temperature for 30 min. The sections were examined under an electron microscope at 80 kV (1200EX; JEOL, Japan).

Effects of inhibitors on degradation of hemoglobin by the aspartic proteinase. The inhibitor studies were performed using hemoglobin as substrate essentially as described previously [28]. The reaction mixture contained 100 µl of 5% hemoglobin, 40 µl of enzyme solution and 360 µl of 0.1 M sodium acetate buffer pH 5.5 with or without each inhibitor. Inhibitors and their concentrations were pepstatin A (1 mM), EDTA (1 mM), N-ethylmaleimide (NEM, 1 mM) and phenylmethylsulfonyl fluoride (PMSF, 1 mM). The reactions were allowed to proceed for 1 hr at 37 °C, and then stopped by addition of 400 µl of 10% trichloroacetic acid (TCA). The amount of TCA-soluble products of the reaction was measured by absorbance at 280 nm.

Purification of a vacuolar processing enzyme. A vacuolar processing enzyme was purified from the soluble matrix fraction of protein-storage vacuoles of castor bean, as described previously [3]. The vacuolar processing enzyme was assayed with capillary electrophoresis as described previously [24]. To determine the optimum pH, the various buffer solutions (each 0.1 M) described below were used.

***In vitro* processing of proprotein precursors in the ER.** Three pairs of maturing endosperm of castor bean were pulse-labeled with TRAN^{35}S-Label (1.9 MBq/endosperm; 37.8 TBq/mmol; ICN Biomedicals Inc., USA) for 1 hr as described previously [3, 22, 29, 30]. The endosperms were rinsed three times with a 10 mM solution of unlabeled methionine and cysteine and were placed on filter paper moistened with water to chase the label. After a chase period of 1 h, the labeled endosperms were homogenized by chopping with a razor blade in a solution of 150 mM Tricine/KOH pH 6.5, 1 mM EDTA and 13% (w/w) sucrose. The homogenate was filtered through one layer of cheesecloth. The filtrate was loaded on a stepwise sucrose gradient and centrifuged in an ultracentrifuge (Hitachi, Japan) at 60000 g for 1 h, as described previously [8, 22, 30]. Both the ER and vacuoles were collected. [^{35}S]-labeled

proprotein precursors of the seed proteins located in the ER fraction were used as substrate for *in vitro* processing. The vacuolar processing enzyme (0.4 munits) was mixed with an aliquot of the ER in 20 µl of 0.1 M sodium acetate pH 5.5, 0.1 M dithiothreitol and 0.1 mM EDTA with or without NEM. Alternatively, the aspartic proteinase (0.4 munits) was mixed with an aliquot of the ER in 20 µl of 0.1 M sodium acetate pH 5.5 with or without pepstatin A. The reactions were incubated for 0, 5 and 30 min at 37 °C. The reaction products were subjected to SDS-PAGE on a 15% acrylamide gel and then analyzed with a Bio-Imaging Analyzer (BAS 2000; Fuji Film, Tokyo, Japan). The identity of each precursor and its corresponding mature form was confirmed by immunoprecipitation analysis or by its molecular mass.

Determination of the cleavage sites of a substrate peptide by the aspartic proteinase. An 18-amino acid peptide was synthesized with a peptide synthesizer (model 430A, Perkin Elmer/Applied Biosystems, Foster City, CA, USA). The sequence, PSLDDEFDLEDDIENPQG, was derived from the sequence containing an internal processing fragment that is post-translationally removed from the proprotein precursor of 2S albumin-2 of *Arabidopsis thaliana* [5]. The peptide (100 nmol) was incubated with the aspartic proteinase in 25 µl of 0.1 M citrate phosphate buffer pH 3.0 for 30 min at 37 °C. The products of the reaction were separated by HPLC. The amino acid composition of each HPLC peak fraction was determined with a Hitachi 835 Amino Acid Analyzer.

Proteolytic activity of the aspartic proteinase. The reaction mixture contained 6 nmol of the substrate peptide and the aspartic proteinase in 5 µl of 20 mM sodium acetate pH 3.0 and 0.1 mM EDTA. The reactions were allowed to proceed for 10 to 30 min at 37 °C. The products of the reaction were subjected to analytical capillary electrophoresis (model 270A; Applied Biosystems, USA) at 30 °C and 20 kV in 10 mM sodium borate pH 9.0. Electrophoresis was monitored in terms of absorbance at 200 nm. One unit of activity was defined as the amount that liberated 1 µmol of the heptapeptide P2 (see Fig. 6) in 1 min at 37 °C.

To determine the optimum pH, the purified aspartic proteinase was incubated with 6 nmol of the substrate peptide in 5 μl of various buffer solutions (each 0.1 M) and 0.1 mM EDTA. Buffer solutions used were glycine/HCl pH 2.5, sodium acetate pH 3.5 to 5.5, citrate phosphate pH 6.0 to 7.0 and Tris/HCl pH 7.5 to 8.5. The reactions were incubated for 30 min at 37 °C and then subjected to capillary electrophoresis.

RESULTS

Aspartic proteinase purified from protein-storage vacuoles of the seeds of castor bean. Aspartic proteinase was purified from the soluble fraction of protein-storage vacuoles of castor bean with pepstatin A-agarose. The activity of the purified enzyme on hemoglobin was completely inhibited by 1 mM pepstatin A, but not by 1 mM of EDTA, NEM or PMSF. The final preparation of the enzyme gave two distinct bands of 48 kDa and 29 kDa on an SDS gel with Coomassie blue staining, as shown in Fig. 1a (lane 2). With a reducing agent, the 48-kDa enzyme was separated into 32-kDa and 16-kDa subunits (Fig. 1a, lane 1), indicating that the 48-kDa aspartic proteinase was composed of two subunits linked by one or more disulfide bonds. In contrast, the reducing agent did not affect the mobility of the 29-kDa subunit on the SDS gel (Fig. 1a, lanes 1 and 2). This suggested that either the 29-kDa subunit or the subunit it binds to lacks a cysteine residue needed for the formation of a disulfide bond. An 11-kDa subunit, a possible counterpart of the 29-kDa subunit, was difficult to detect on the gel with Coomassie blue staining. This might be due to a low stainability of the molecule.

This result raised the question of whether the 29-kDa subunit is another isoform of aspartic proteinases or a degradation product of the 32-kDa subunit of the enzyme. To answer this question, the N-terminal amino-acid sequences of the 32-kDa and 29-kDa subunits were determined. The N-terminal sequence of the 32-kDa subunit (DAFDTDIVALKNYLDAQY) was different from that of the 29-kDa subunit

(GDSKDTDIVALKNYLDAQY). The N-terminal Asp-1 of the 32-kDa subunit corresponded to the second amino acid of the 29-kDa subunit. Ala-2 and Phe-3 of the 32-kDa subunit were replaced with Ser-3 and Lys-4 in the sequence of the 29-kDa subunit, respectively. This result strongly suggested that two isoforms of aspartic proteinase exist in the endosperm of castor bean: one is linked by one or more disulfide bond(s) and the other is not.

The N-terminal amino acid sequences of the subunits were aligned with sequences of precursors of the aspartic proteinases of a dicot (pumpkin, this study), a monocot (barley) [18] and yeast [19], as shown in Fig. 1b. The precursor sequence of the pumpkin enzyme was deduced from the nucleotide sequence of a cDNA that was isolated from a library [31] made from the maturing pumpkin seeds. The deduced precursor is 66% identical in amino acid sequence to the aspartic proteinase of a monocot (barley).

The N-terminal amino acid sequences of the subunits of castor bean aspartic protease (underlined in Fig. 1b) more closely match the sequence of pumpkin than that of barley. The N-terminal sequence of the 16-kDa subunit, DAMXSTX (X, no amino acid detected), starts at Asp-384 of the precursor of pumpkin aspartic protease (underlined Fig. 1b). After a co-translational cleavage of the signal peptide at the C-terminal side of Ala-25 [32], post-translational cleavages to remove both the N-terminal propeptide and an internal propeptide take place to generate the heterodimeric enzyme composed of 32-kDa and 16-kDa subunits.

The post-translational removal of the N-terminal propeptide to generate the 29-kDa subunit might occur at a site one residue preceding the cleavage site that generates the 32-kDa subunit. Runeberg-Roos *et al.* suggested that an NPLR sequence just before the cleavage site of the barley enzyme may be a vacuolar targeting signal (double-underlined in Fig. 1b)[15] . However, the pumpkin enzyme has no NPLR sequence in the same propeptide region (Fig. 1b). The finding suggests another targeting signal might be exist in the proenzyme sequence.

Cellular distribution of the aspartic proteinase and associated changes in its level during development. To determine the intracellular localization of aspartic proteinase, we prepared antibodies against the enzyme purified from castor bean and performed both immunoblot and immunocytochemical analyses with the antibodies. Protein-storage vacuoles were highly purified from dry seeds. A homogeneous band of 48 kDa was detected on the immunoblot of the protein-storage vacuoles under non-reducing conditions (Fig. 2, lane 4). Each protein-storage vacuole of castor bean contains a crystalloid composed of 11S globulin, a major storage protein [33]. The protein-storage vacuoles were burst and separated by centrifugation into two suborganellar fractions: a soluble matrix and the insoluble crystalloids plus membranes [31]. Under the centrifugation conditions, *Ricinus communis* agglutinin (RCA), ricin and 2S albumin were precipitated together with 11S globulin (Fig. 2, lane 3). The immunoblot of the two fractions showed that the aspartic proteinase was localized in the soluble matrix fraction of the protein-storage vacuoles, but not in the insoluble fraction (Fig. 2, lanes 5 and 6).

To further localize the aspartic proteinase in maturing seeds of castor bean, we performed an immunocytochemical analysis with antibodies against either the aspartic proteinase or 11S globulin. Fig. 3a shows that the aspartic proteinase was localized in the matrix region of the protein-storage vacuoles, but not in the crystalloid. This is consistent with the results obtained with protein-storage vacuoles of dry seeds (Fig. 2, lanes 5 and 6). In contrast, 11S globulin was distributed in both the vacuolar matrix and crystalloid in the maturing endosperm (Fig. 3b). These results indicated that the aspartic proteinase was localized in the vacuolar matrix together with a major storage protein during seed maturation.

The co-localization of the aspartic proteinase with a storage protein suggested the possibility that the enzyme may be involved in maturation of the storage proteins. Developmental changes in the level of the enzyme in the endosperm tissues of the castor bean were examined during seed maturation and after seed germination. Storage proteins of castor bean are actively synthesized and accumulated at 30 to 40 days after anthesis [22],

as shown in Fig. 4a (stage III). The amount of the aspartic proteinase increased at stage II prior to the accumulation of the storage proteins (Fig. 4b, c). In contrast, the amount of the enzyme decreased after seed germination (Fig. 4b, c). The reduction of the enzyme was associated with a rapid degradation of the storage proteins in the 2 days after germination (Fig. 4a). This result suggested that the aspartic proteinase might play some role in the maturation, rather than the degradation, of the storage proteins.

***In vitro* processing of proprotein precursors of storage proteins.** To examine the ability of the aspartic proteinase to process authentic proprotein precursors, we prepared an ER fraction, as the ER is where the proproteins are synthesized in maturing castor bean seeds [22]. At mid maturation of the castor bean, the [^{35}S]-labeled endosperm were fractionated by sucrose density gradient centrifugation. The ER was prepared from the pulse-labeled endosperm and the protein-storage vacuoles were prepared from the pulse-chased endosperm. The protein-storage vacuoles contained mature forms of storage proteins, such as 2S albumin, the small subunit of 11S globulin and RCA plus ricin (Fig. 5, lane 1). The labeled mature proteins were used as size markers of each storage protein on a Bio-Imaging Plate. In contrast, the ER fraction contained their respective proproteins, such as pro2S albumin, proglobulin and proRCA plus proricin (Fig. 5, lane 2). These labeled proproteins were used as substrates for an *in vitro* proteolytic processing assay.

After these three labeled proproteins, which are located only in the ER, were incubated without any enzyme, no endogenous proteolysis was observed (Fig. 5, lane 3). The vacuolar processing enzyme was reported to be synthesized on the rER as an inactive form [4]. The aspartic proteinase synthesized on the rER might also be inactive. During incubation of the labeled proproteins with the vacuolar processing enzyme up to 30 min, the radioactivity in all three proproteins decreased, while there was a concomitant increase in the radioactivity of the mature proteins (Fig. 5, lanes 2, 4 and 5). The size of each product was the same as the size found in the vacuoles (Fig. 5, lane 1). Processing of the three precursors was inhibited in the presence of 2 mM NEM (Fig. 5, lane 6), which is an

inhibitor of the thiol endopeptidase of the vacuolar processing enzyme [3]. On the other hand, no processing of the labeled proproteins was observed by addition of the aspartic proteinase (Fig. 5, lanes 7 and 8). This indicated that the vacuolar processing enzyme mediated the conversion of proproteins into their mature forms, but the aspartic proteinase had no such ability.

***In vitro* cleavage of an internal propeptide of pro2S albumin.** Pro2S albumins are reported to contain a linker peptide between the two subunits of the mature protein. The internal propeptide must be cut out during seed maturation. The removal of the propeptide might occur after the processing of proproteins by the vacuolar processing enzyme. To further examine the involvement of the aspartic proteinase in the maturation of storage proteins, we performed an *in vitro* excision of the internal propeptide of 2S albumin. *Arabidopsis* 2S albumin-2 is one of the most characterized storage proteins with respect to post-translational cleavages [5]. Thus, we synthesized an 18-amino-acid peptide that contained a sequence of the internal propeptide of the *Arabidopsis* pro2S albumin-2 [5].

During incubation of the internal propeptide with the aspartic proteinase for 30 min at pH 3.0, most of the peptide was hydrolyzed to produce five peptide fragments, as shown in Fig. 6a. The amino acid composition of each peptide fragment revealed that the enzyme cleaved the internal propeptide at three sites (Fig. 6b, c). The first cleavage occurred between Phe-7 and Asp-8 to produce the P2 and P5 peptides (Fig. 6c). The second cleavage occurred between Leu-9 and Asp-10 to produce the P3 and P4 peptides (Fig. 6c). The third cleavage occurred between Asp-12 and Ile-13 to produce the P1 peptide, which might be a degradation product of P4 and P5 (Fig. 6c). This suggested that the aspartic proteinase cleaved peptide bonds in which one of the amino acid residues had a bulky hydrophobic side chain. This result also suggested that the aspartic proteinase might be involved in the degradation of the internal propeptide of 2S albumin after conversion of pro2S albumin into two subunits by the vacuolar processing enzyme. However, these cleavages of the internal propeptide were not observed at pH 5.5, which is similar to the

intravacuolar pH of castor bean endosperm [34]. Fig. 7 shows the pH-dependency of the activity of both the aspartic proteinase and the vacuolar processing enzyme. The aspartic proteinase was able to cleave the peptide effectively below pH 4.0, and the optimum was pH 3.0. In contrast, the vacuolar processing enzyme showed little activity at pH 3.0, and the optimum pH was 5.5. These results suggested that the activity of the aspartic proteinase was very low in the vacuoles, and that the cleavage of the internal propeptide might occur slowly (discussed below).

DISSCUSION

Co-localization of the aspartic proteinase with the vacuolar processing enzyme in the protein-storage vacuoles. Immunocytochemical analysis demonstrated that the aspartic proteinase was localized in the matrix region of the protein-storage vacuoles in maturing endosperm of castor bean (Fig. 3). This indicates that the enzyme is co-localized with the vacuolar processing enzyme [4, 24]. Increases in the levels of both enzymes during seed maturation (Fig. 4) [24] suggested that these enzymes might be cooperatively involved in the maturation of seed storage proteins. Proprotein precursors to seed proteins that are translocated into the vacuoles must be exposed to the two proteolytic enzymes.

A precursor sequence to a dicot aspartic proteinase was deduced from the isolated cDNA sequence (Fig. 1). It seemed likely that the precursor synthesized on rER was transported to the protein-storage vacuoles and then converted into the mature form in the vacuoles. Previously, we reported that a vacuolar processing enzyme is synthesized on the ER as an inactive precursor and a removal of the propeptides of the precursors in the vacuoles is necessary to activate the enzyme [2, 24]. It remains to be solved whether the precursor to the plant aspartic proteinase has a proteolytic activity.

The initial proteolytic processing of proproteins is mediated by the vacuolar processing enzyme. For an *in vitro* processing assay of the two enzymes, we prepared an ER fraction from the maturing castor bean seeds and used the authentic proproteins located in this fraction (including pro2S albumin, proglobulin and proRCA/proricin) as substrates. All the proproteins were converted into their mature size by the purified vacuolar processing enzyme, but not by the purified aspartic proteinase (Fig. 5). D'Hondt *et al.* reported that *Brassica* aspartic proteinase could process *Arabidopsis* pro2S albumin-2 at pH 3.5 [14]. They synthesized pro2S albumin *in vitro* using rabbit reticulocyte lysate and then purified it by making two cuts with TCA (5% and 10%). The possibility that the substrate was denatured by this treatment cannot be excluded. The pH of the reaction (3.5) that they used is much lower than the intravacuolar pH (discussed below).

The protein-storage vacuoles isolated from maturing pumpkin seeds contained both a vacuolar processing enzyme [30] and an aspartic proteinase (data not shown). We previously reported that the vacuolar extract could convert proglobulin into the mature forms, and that the conversion could be inhibited by thiol reagents, such as NEM, *p*-mercuribenzoate and monoiodic acetic acid, but not by pepstatin A [30]. These results indicate that the vacuolar processing enzyme acts on the conversion of proproteins into their mature forms before further removal of propeptides.

In vivo analysis by pulse-chase experiments of maturing pumpkin seeds revealed that the proprotein processing occurs immediately after the translocation of the proproteins into the vacuoles [8]. The activity of the vacuolar processing enzyme from maturing endosperm of castor bean is sufficiently high to convert proglobulin and pro2S albumin to their mature forms within 30 min *in vitro* [3]. This supports the hypothesis that the conversion by the vacuolar processing enzyme might precede the removal of the propeptides.

Secondary proteolytic removal of propeptides is mediated by the aspartic proteinase. The vacuolar processing enzyme can recognize exposed two asparagine residues on the molecular surface of pro2S albumin and cleave the peptide bond on the C-terminal side of each asparagine residue [8]. Thus, the enzyme could split *Arabidopsis* pro2S albumin-2 to produce three domains, an N-terminal propeptide, a small subunit with a 16-amino-acid propeptide and a large subunit of the mature protein [5, 8]. Further proteolysis to remove the 16-amino-acid propeptide from the small subunit must occur in the vacuoles. The purified aspartic proteinase could hydrolyze a synthetic peptide containing the internal propeptide sequence at three sites (Fig. 6). This result indicated that the aspartic proteinase might be involved in the removal and degradation of the internal peptide of pro2S albumin-2.

The present study revealed that the aspartic proteinase cleaved peptide bonds in which one of the amino acid residues had a bulky hydrophobic side chain, but not peptide bonds between hydrophilic amino acids (Fig. 6). This was consistent with the substrate specificity of barley aspartic proteinase [35] and mammal aspartic proteinase [36], that were reported to cleave peptide bonds between Asp and Tyr and between amino acid residues with large hydrophobic side chains. In contrast, *Brassica* aspartic proteinase cleaved the same peptide as we used between Asp-11 and Asp-12, but not between Leu-9 and Glu-10 or between Asp-12 and Ile-13 [14]. Although the substrate specificity of the plant aspartic proteinases is still obscure, the proteolytic enzymes might have a substrate specificity that could be suitable for the degradation of propeptides. It therefore seemed that proproteins of 2S albumins might be limitedly cleaved by the vacuolar processing enzyme with a very strict substrate specificity, and then small propeptide domains might be further degraded by the aspartic proteinase to complete their protein maturation. It should be noted that the optimum pH of the aspartic proteinase is 3.0, and proteolytic activity on the peptide is very low above pH 4.0 under the conditions described above (Fig. 7). Nishimura reported that the intravacuolar pH of castor bean endosperm is 5.0 [34]. This indicates that the aspartic proteinase could not show its maximum activity in the protein-storage vacuoles.

A similar vacuolar processing system mediated by both the vacuolar processing enzyme and the aspartic proteinase might function in vegetative vacuoles in various plant organs.

We have proposed that the same type of proteolytic enzyme is involved in the post-translational processing of many vacuolar proteins with significant variability in molecular structure [2, 3]. The activity of the vacuolar processing enzyme can be found not only in seeds but also in other vegetative organs, such as hypocotyls, roots and mature leaves [24]. It has been reported that proteinase inhibitors of tomato leaves [37] and tobacco stigmas [38], and chitinase from tobacco leaves and cultured cells [39] are processed post-translationally on the C-terminal side of an asparagine residue to yield the mature forms. Recently, we isolated three genomic DNAs for vacuolar processing enzymes (αVPE, βVPE and γVPE) that are expressed in specific organs in *Arabidopsis* [40, 41]. βVPE is specific to seed, while αVPE and γVPE are specific to vegetative organs [40, 41]. Thus, a similar cysteine proteinase is widely distributed in plant organs and plays a crucial role in maturation of a variety of proteins in plant vacuoles.

The aspartic proteinase is also found in various tissues of plants [42]. We have isolated two isoforms of the aspartic proteinase (Fig. 1). Paris *et al.* discussed that a single aspartic proteinase has two kinds of vacuolar targeting signals in the proenzyme, an NPLR sequence for vegetative vacuoles and an internal propeptide sequence for protein-storage vacuoles [16]. However, the NPLR sequence is not conserved in pumpkin aspartic proteinase (Fig. 1b). Two cDNAs for aspartic proteinase were also isolated from rice (accession numbers D32144 and D32165). It is likely that more than two homologues of the enzyme exist in plants, as vacuolar processing enzyme homologues do. Both the aspartic proteinase and the vacuolar processing enzyme function in the maturation of proproteins of vacuolar proteins and/or degradation of some proteins in the seed and vegetative organs of higher plants.

Footnotes

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‖ The nucleotide sequence data of the pumpkin aspartic proteinase will appear in the GSDB, DDBJ, EMBL, NCBI nucleotide sequence databases with the accession number xxxx.

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FIGURE LEGENDS

Fig. 1. Structure and sequence of an aspartic proteinase purified from protein-storage vacuoles of castor bean. (a) SDS-PAGE gel showing an aspartic proteinase purified from the soluble matrix fraction of the protein-storage vacuoles in the presence (lane 1) or absence (lane 2) of 2-mercaptoethanol. Separated proteins were stained with Coomassie blue. Molecular masses of subunits of the purified enzyme are shown on the left and molecular masses of marker proteins (lane M) are shown on the right in kDa. (b) Alignment of the N-terminal sequences of the 32-, 29- and 16-kDa subunits of the aspartic proteinase (indicated by underlining) with sequences of precursors to the aspartic proteinases of a dicotyledonous plant (pumpkin), a monocotyledonous plant (barley) [18] and yeast [19]. The primary sequence of the pumpkin enzyme was deduced from the nucleotide sequences of the isolated cDNA. An NPLR sequence, a possible signal for targeting to vacuoles, is found in the N-terminal propeptide of the barley enzyme (indicated by double-underlining), but not in that of the pumpkin enzyme. The closed triangle indicates a putative cleavage site of a signal peptide and open triangles indicate possible sites of post-translational cleavages to generate a mature enzyme. Closed circles indicate two aspartic acid residues at the active site of the enzyme. Open circles indicate possible N-linked glycosylation sites. Boxed residues indicate identical amino acids, and shaded residues indicate homologous amino acids.

Fig. 2. SDS-PAGE gel showing suborganellar localization of the aspartic proteinase in protein-storage vacuoles of endosperm of castor bean. Lanes 1 and 4, protein-storage vacuoles; lanes 2 and 5, soluble portion of protein-storage vacuoles; lanes 3 and 6, insoluble portion of protein-storage vacuoles. R, RCA plus ricin; G, 11S globulin; 2S, 2S albumin. **CBB**, gel stained with Coomassie blue; **anti-AP**, immunoblot analysis with anti-aspartic proteinase antibodies. Samples were electrophoresed without 2-mercaptoethanol. Molecular masses of marker proteins are shown on the right in kDa.

Fig. 3. Immunoelectron micrographs showing localization of the aspartic proteinase in maturing castor bean endosperm. Ultrathin sections of the endosperm at the middle stage of seed maturation were incubated with either anti-aspartic proteinase antibodies (a) or anti-11S globulin antibodies (b). VM, vacuolar matrix; VC, vacuolar crystalloid; Mt, mitochondrion; L, lipid body. Bars = 1 μm.

Fig. 4. Developmental changes in the amount of the aspartic proteinase and the accumulation of storage proteins in the endosperm of castor bean during seed maturation and germination. (a) SDS-PAGE gel of endosperm tissues freshly harvested from maturing seeds at the early (I), middle (II) and late (III) stages, from dry seeds (0) and from 2-, 4-, 6- and 8-day-old seedlings. The major storage proteins, RCA plus ricin (**R**) and 11S globulin (**G**) accumulated in dry seeds (0). Separated proteins in the presence of 2-mercaptoethanol were stained with Coomassie blue. Molecular masses of marker proteins (lane M) are shown on the left in kDa. (b) Immunoblot analysis of the same samples as in (a) using anti-aspartic proteinase antibodies (anti-AP). SDS-PAGE was carried out in the absence of 2-mercaptoethanol. (c) Levels of the enzyme in (b) measured with a densitometer. The amount of enzyme is expressed as a percentage of the maximum value.

Fig. 5. *In vitro* proteolytic processing of [^{35}S]proproteins located in the ER from the maturing endosperm of castor bean. The endosperm of castor bean at the middle stage of seed maturation was pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine for 1 hr and subsequently chased with cold methionine and cysteine for 2 hr. ER was prepared from the endosperm immediately after the pulse and vacuoles were prepared form the pulse-chased endosperm by sucrose density gradient centrifugation. Lane 1, vacuole fraction showing major [^{35}S]mature seed proteins, including 2S albumin (**2S**), the small subunit of 11S globulin (**G**) and RCA plus ricin (**R**); lanes 2-9, ER fractions. Lanes 2 and 3, ER incubated for 0 and 30 min without enzyme showing the

[^{35}S]proprotein precursors of the seed proteins: pro2S albumin (**p2S**), proglobulin (**pG**) and proproteins to RCA and ricin (**pR**); lanes 4 and 5, ER incubated with vacuolar processing enzyme (**VPE**, 0.4 mU) for 5 and 30 min; lane 6, ER incubated with VPE for 30 min with 2 mM NEM, an inhibitor of vacuolar processing enzymes; lanes 7 and 8, ER incubated with aspartic proteinase (**AP**, 0.4 mU) for 5 and 30 min; lane 9, ER incubated with AP for 30 min with 0.1 mM pepstatin A, an inhibitor of aspartic proteinases. Incubations were carried out at 37 °C. Reaction products were subjected to SDS-PAGE and visualized with a Bio-Imaging Analyzer (BAS 2000; Fuji Film, Tokyo, Japan).

Fig. 6. Cleavage products of aspartic proteinase acting on an 18-amino-acid synthetic peptide corresponding to an internal propeptide fragment of Arabidopsis 2S albumin. This fragment is post-translationally removed to form 2S albumin-2 [5]. (a) Capillary electrophoresis chromatograms of the substrate (S) before and after cleavage. Five cleaved products (P1, P2, P3, P4 and P5) were produced by the enzyme. (b) Amino acid compositions of the substrate and the five cleavage products. Values are expressed relative to the number of moles of proline. The numbers in parentheses are the estimated numbers of each amino acid rounded off to the nearest integer. n. d. = not detected. (c) Deduced amino acid sequences of the five cleavage products. The sequence of the substrate peptide is shown at the top. Three cleavage sites of the peptide by the aspartic proteinase are indicated by closed triangles.

Fig. 7. Comparison of optimum pH values of the aspartic proteinase (closed circles) and the vacuolar processing enzyme (closed squares). Both enzymes were purified from the protein-storage vacuoles of castor bean. The activity of the aspartic proteinase was determined with the synthetic peptide shown in Fig. 6. The activity of the vacuolar processing enzyme was measured as described previously [24]. The reactions were run for 30 min at 37 °C. Reaction products were detected with capillary electrophoresis.

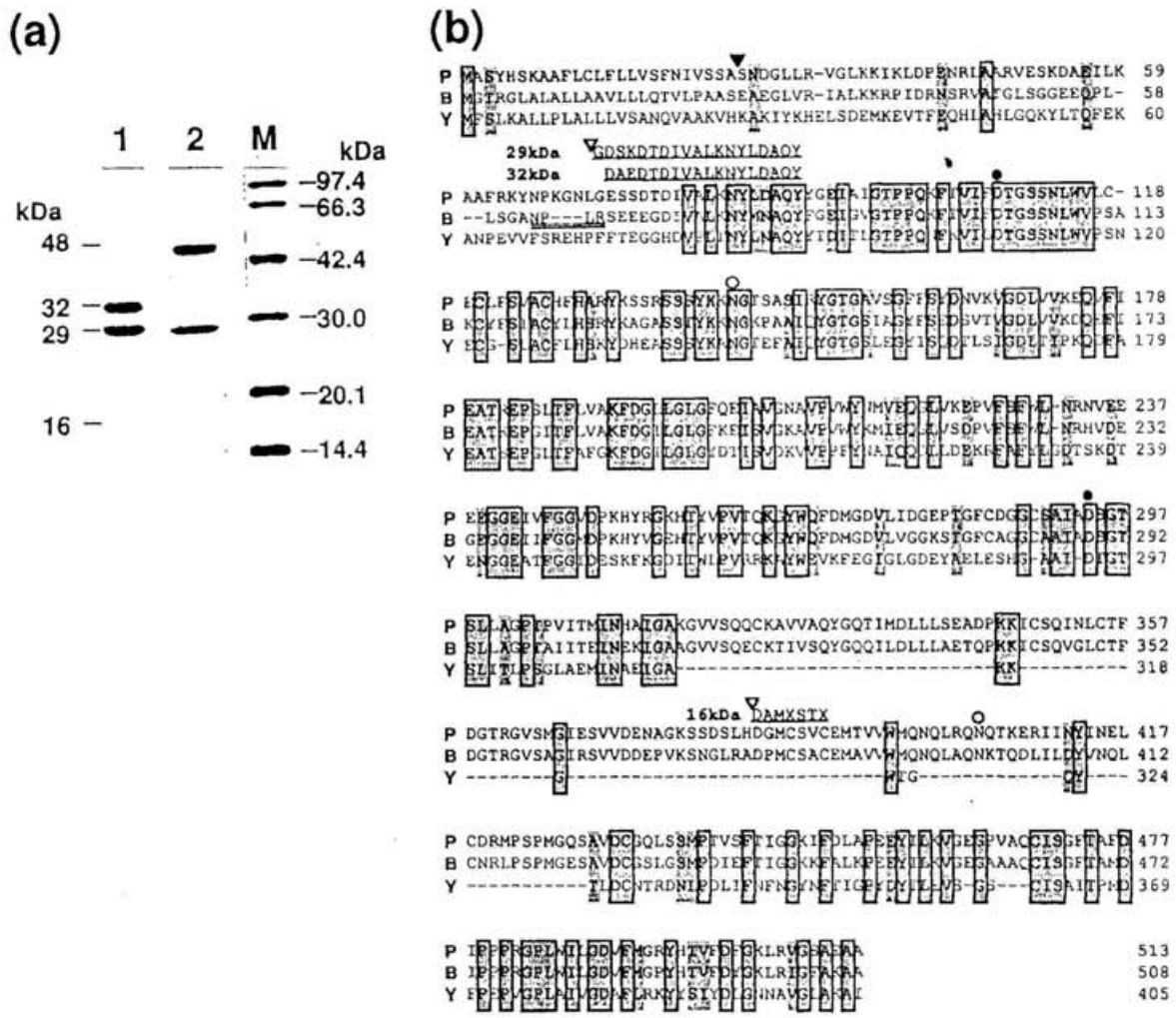
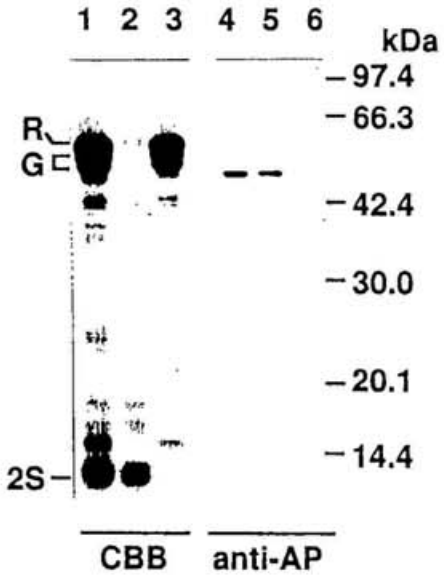
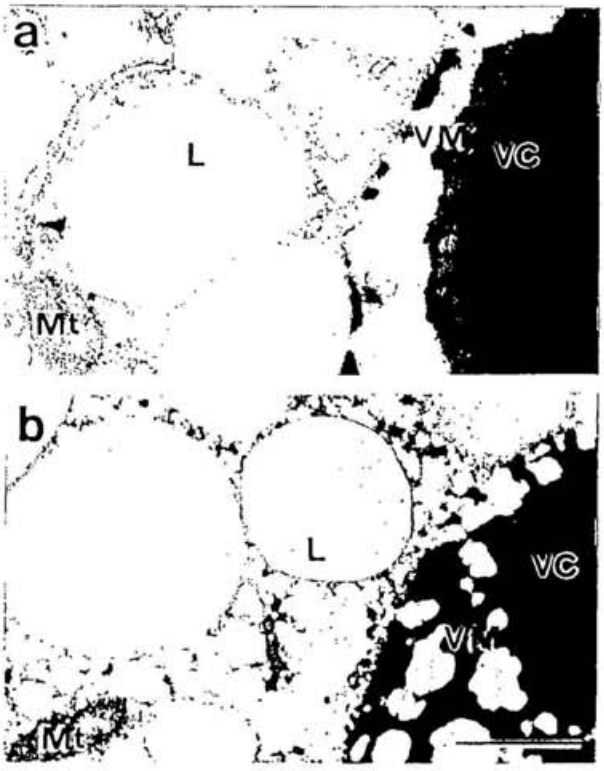


Fig. 1. Hiraiwa *et al.*



ig. 2. Hiraiwa *et al.*



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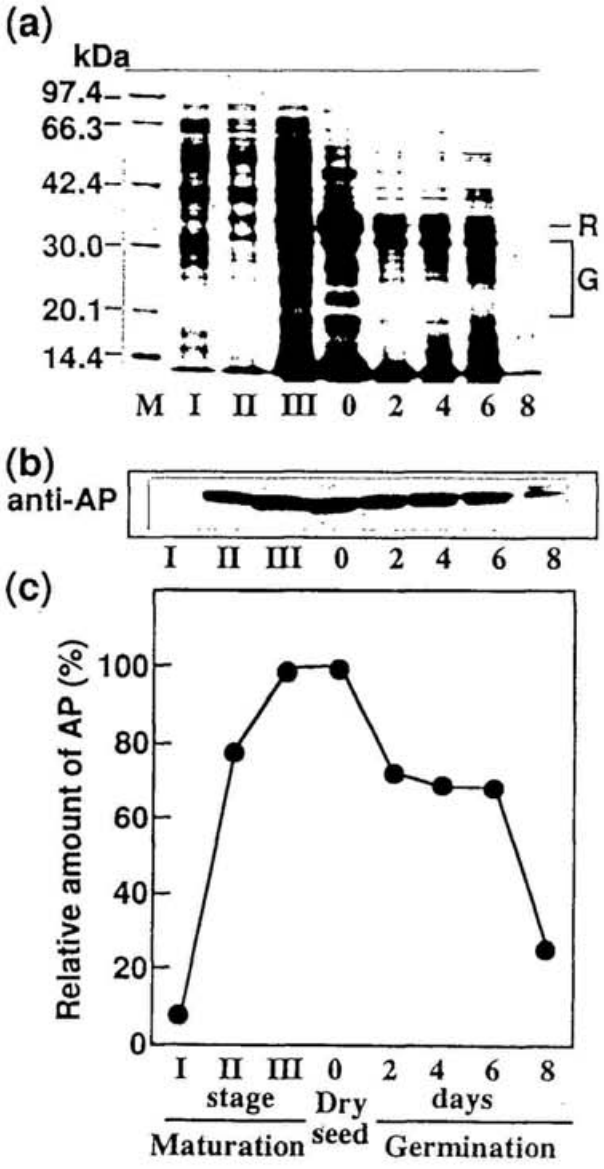

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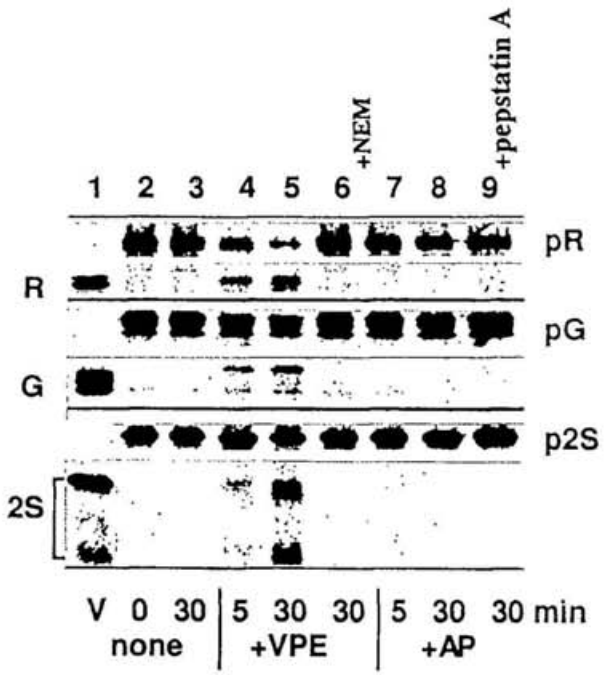
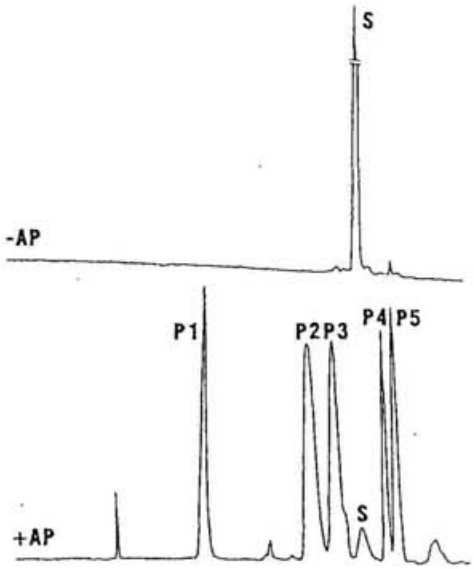


Fig. 5. Hiraiwa *et al.*

(a)



(b)

	Amino acid composition (mol)					
	S	P1	P2	P3	P4	P5
Asp	6.20 (6)	1.11 (1)	2.39 (2)	3.39 (3)	2.89 (3)	4.08 (4)
Ser	0.92 (1)	n. d.	0.97 (1)	0.84 (1)	n.d.	n.d.
Glu	4.26 (4)	1.97 (2)	1.24 (1)	1.02 (1)	2.89 (3)	2.44 (3)
Pro	2.00 (2)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Gly	1.05 (1)	1.08 (1)	n. d.	n. d.	0.88 (1)	1.05 (1)
Ile	2.08 (2)	0.85 (1)	n. d.	n. d.	0.85 (1)	0.82 (1)
Lue	2.08 (2)	n. d.	1.05 (1)	2.09 (2)	0.29 (0)	1.05 (1)
Phe	1.02 (1)	n. d.	1.02 (1)	1.05 (1)	n. d.	n. d.

(c)

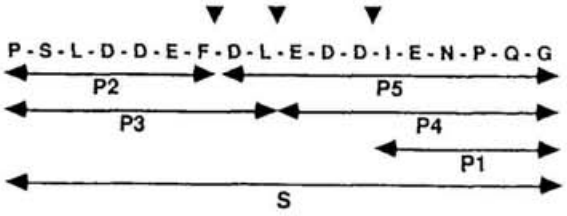


Fig. 6. Hiraiwa *et al.*

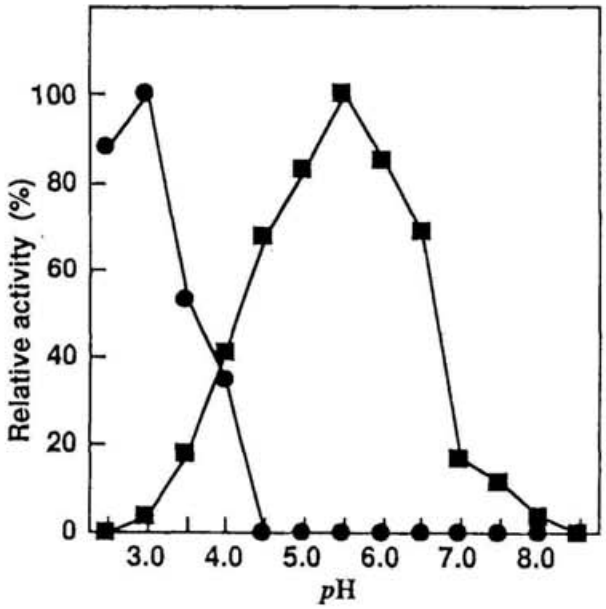


Fig. 7. Hiraiwa *et al.*

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Rapid report

Vacuolar Processing Enzyme of Soybean That Converts Proproteins to the Corresponding Mature Forms

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A vacuolar processing enzyme was detected in soybean protein bodies. A 39-kDa immunoreactive polypeptide obtained by chromatography on a hydroxyapatite column processed both a decapeptide substrate and proproteins. A cDNA was isolated for a 55-kDa protein with 71% identity to the castor bean vacuolar processing enzyme.

Key words: Cysteine proteinase — 11S Globulin — Proprotein processing — Protein body — Soybean (*Glycine max*) — Vacuolar processing enzyme.

Soybean (*Glycine max*) glycinin, which belongs to the 11S globulin family, is a well known storage protein (Higgins 1984, Nielsen et al. 1989). Proglobulins, the protein precursors of 11S globulins, are synthesized on the endoplasmic reticulum and are then transported to protein-storage vacuoles, where they are processed proteolytically to yield the corresponding mature forms during seed maturation (Akazawa and Hara-Nishimura 1985, Fukasawa et al. 1988, Hara-Nishimura et al. 1985). There are two reports of the purification of processing enzymes that convert proglycinin to glycinin in maturing soybean seeds (Scott et al. 1992, Muramatsu and Fukazawa 1993). However, there is a big discrepancy in terms of molecular mass between the two reported enzymes (as discussed below).

Proglycinin is cleaved on the C-terminal side of an asparagine residue to yield mature glycinin (Nielsen et al. 1989). We succeeded previously in purifying a 37-kDa cysteine proteinase from castor bean (*Ricinus communis*) that specifically cleaves a peptide bond on the C-terminal side of asparagine residues in different proproteins, including proglobulins, to generate the corresponding mature forms (Hara-Nishimura et al. 1991). To examine whether a processing enzyme similar to the castor bean enzyme plays a role in processing of proproteins in soybean, we isolated a cDNA for a soybean homologue of the castor bean enzyme and characterized it.

Poly(A)$^+$RNA was prepared from maturing seeds of soybean by SDS-phenol extraction and chromatography on oligo(dT)-cellulose. A cDNA library in pBluescript II SK+ (Stratagene, La Jolla, CA, U.S.A.) was constructed as described previously (Mori et al. 1991). Two degenerate primers, 5′-CA(T/C)CA(G/A)GC(G/A/T/C)GA(T/C)GT(G/A/T/C)TG(T/C)CA(T/C)GC-3′ and 5′-CC(G/A/T/C)GG(G/A/T/C)CC(G/A/T/C)CC(G/A)TG(G/A)TC-3′, were synthesized (indicated by two arrows in Fig. 2). Their sequences were based on the amino acid sequences, HQADVCHA and DHGGPG, that are conserved between the processing enzyme of castor bean (Hara-Nishimura et al. 1993b) and an enzyme from *Schistosoma* (Klinkert et al. 1989). Amplification by the polymerase chain reaction (PCR) was performed using these primers and plasmid DNA of the cDNA library. Then the cDNA library was screened with the 320-bp product of PCR as probe. The cloning procedures were essentially the same as reported previously (Hara-Nishimura et al. 1993b). The positive clone with the longest insert of, namely, 1.7 kb was isolated and designated sVPE. Sequencing of both strands of the insert was performed with a DNA sequencer (model 373A; Applied Biosystems Inc., Foster City, CA, U.S.A.) using T3 and T7 fluorescent primers in accordance with the manufacturer's directions.

Protein bodies were isolated from dry seeds of soybean and castor bean by the glycerol method, as described

Abbreviation: PCR, polymerase chain reaction.

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previously (Hara-Nishimura et al. 1982). After lysis of the isolated protein bodies in 50 mM sodium acetate, pH 5.5, each homogenate was centrifuged at 100,000 × *g* for 20 min at 4°C. The supernatant was dialyzed against the sodium acetate buffer at 4°C and centrifuged again to remove insoluble globulins. The supernatant was used as a matrix fraction of protein bodies and was subjected to SDS-PAGE and immunoblotting analysis. An antibody was raised against the vacuolar processing enzyme of castor bean that had been expressed in *E. coli* (Hara-Nishimura et al. 1993b, Hiraiwa et al. 1993).

The matrix fraction of soybean protein bodies was applied to a hydroxyapatite column (7.5 mm i.d. × 7.5 cm, HA-1000; Tosoh, Kyoto, Japan) which had been equilibrated in 10 mM sodium phosphate, pH 6.0, on an FPLC system (Pharmacia, Pleasant Hill, CA, U.S.A.). After extensive washing of the column with the same buffer, proteins were eluted with a linear gradient of sodium phosphate, pH 6.0, from 0.01 M to 0.5 M. Each fraction after chromatography was subjected to SDS-PAGE with subsequent staining with Coomassie blue and immunoblotting analysis. Vacuolar processing activity in each fraction was measured as described previously (Hara-Nishimura et al. 1991, Hiraiwa et al. 1993). We used a chemically synthesized decapeptide, SESENGLEET, as substrate. Its sequence was derived from the sequence around the processing site of pumpkin proglobulin. One unit of activity was defined as the activity that processed 1 μmole of substrate per min under the conditions of the reaction.

The processing activity with proproteins as substrates was examined essentially by the methods described previously (Hara-Nishimura and Nishimura 1987, Hara-Nishimura et al. 1991). Proproteins were prepared from dense vesicles that had been isolated from the pulse-labeled endosperm of maturing seeds of castor bean (Hara-Nishimura and Nishimura 1987, Hara-Nishimura et al. 1993a). Pulse labeling was performed with [^{35}S]methionine and [^{35}S]cysteine together. The reaction mixture, containing labeled proproteins and the soybean enzyme that had been purified on the hydroxyapatite column, was incubated in the presence or absence of *N*-ethylmaleimide for 60 min at 37°C and then subjected to SDS-PAGE subsequent fluorography.

Figure 1 shows the nucleotide sequence and predicted amino acid sequence of sVPE cDNA for the soybean homologue of the processing enzyme. The clone encodes a protein of 495 amino acids with a molecular mass of 55,162 Da. As shown in Figure 2, the 55-kDa precursor is 71% identical to the vacuolar processing enzyme of castor bean (Hara-Nishimura et al. 1993b), 32% identical to the putative cysteine proteinase in the human parasite *Schistosoma mansoni* (Klinkert et al. 1989) and 33% identical to the putative cysteine proteinase in *Schistosoma japonicum* (accession number S31908 in the peptide sequence database of PIR, Protein Information Resouce), respectively. The vacuolar processing enzymes of both soybean and castor bean have properties characteristic of cysteine proteinases (Fig. 5, Hara-Nishimura and Nishimura 1987, Hara-Nishimura et al. 1991). However, these vacuolar processing enzymes have no significant sequence similarity to known cysteine proteinases, such as papain (Cohen et al. 1986) and barley aleurain (Rogers et al. 1985). This observation suggests that plant vacuolar processing enzymes and the enzymes of *Schistosoma* belong to a new class of cysteine proteinases.

Hydropathy analysis showed that the 55-kDa precur-

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                -40  gtgagtgaccgaqtgagtttgtttttctcagctgatatat    -1
                      ***          ***
ATGGCGCTTGATCGCTCCATTATAAGCAAAACGACGTGGTACAGCGTCGTATTATGGATG     60
M  A  L  D  R  S  I  I  S  K  T  T  W  Y  S  V  V  L  W  M      20
ATGGTGGTGCTGGTGAGAGTGCACGGTGCAGCCGCGAGGCCGAACCGGAAGGAGTGGGAC  120
M  V  V  L  V  R  V  H  G  A  A  A  R  P  N  R  K  E  W  D     40
TCAGTCATAAAGTTACCGACTGAACCGGTGGATGCTGACTCGGATGAAGTGGGAACACGA  180
S  V  I  K  L  P  T  E  P  V  D  A  D  S  D  E  V  G  T  R     60
TGGGCGGTTCTCGTGGCTGGTTCAAACGGCTACGGAAACTACAGGCATCAAGCAGATGTG  240
W  A  V  L  V  A  G  S  N  G  Y  G  N  Y  R  H  Q  A  D  V     80
TGCCATGCGTACCAGTTGCTGATAAAAGGTGGACTAAAAGAAGAGAACATAGTGGTGTTT  300
C  H  A  Y  Q  L  L  I  K  G  G  L  K  E  E  N  I  V  V  F    100
ATGTACGATGACATAGCTACCAACGAGTTGAATCCTAGACATGGAGTCATCATCAACCAC  360
M  Y  D  D  I  A  T  N  E  L  N  P  R  H  G  V  I  I  N  H    120
CCTGAGGGAGAAGATCTGTATGCTGGTGTTCCTAAGGATTACACCGGTGATAATGTGACG  420
P  E  G  E  D  L  Y  A  G  V  P  K  D  Y  T  G  D  N  V  T    140
ACGGAGAACCTCTTTGCTGTTATTCTTGGAGACAAGAGTAAATTGAAGGGAGGAAGTGGC  480
T  E  N  L  F  A  V  I  L  G  D  K  S  K  L  K  G  G  S  G    160
AAAGTGATCAACAGCAAACCCGAGGACAGAATATTTATATACTACTCTGATCATGGAGGT  540
K  V  I  N  S  K  P  E  D  R  I  F  I  Y  Y  S  D  H  G  G    180
CCTGGAATACTTGGGATGCCAAACATGCCATACCTTTATGCCATGGATTTTATTGATGTC  600
P  G  I  L  G  M  P  N  M  P  Y  L  Y  A  M  D  F  I  D  V    200
TTGAAGAAGAAACATGCATCTGGAAGTTACAAGGAGATGGTTATATACGTGGAAGCTTGT  660
L  K  K  K  H  A  S  G  S  Y  K  E  M  V  I  Y  V  E  A  C    220
GAAAGTGGGAGCGTGTTTGAGGGTATAATGCCTAAGGATCTGAATATTTATGTCACAACT  720
E  S  G  S  V  F  E  G  I  M  P  K  D  L  N  I  Y  V  T  T    240
GCATCAAATGCACAAGAGAATAGTTGGGGGACTTATTGTCCTGGAATGGATCCTTCTCCA  780
A  S  N  A  Q  E  N  S  W  G  T  Y  C  P  G  M  D  P  S  P    260
CCTCCAGAGTACATCACTTGCCTAGGGGATTTGTACAGCGTTGCTTGGATGGAAGATAGT  840
P  P  E  Y  I  T  C  L  G  D  L  Y  S  V  A  W  M  E  D  S    280
GAGGCTCACAATCTAAAAAGGGAATCCGTGAAACAACAATACAAATCGGTAAAGCAACGG  900
E  A  H  N  L  K  R  E  S  V  K  Q  Q  Y  K  S  V  K  Q  R    300
ACTTCAAATTTCAACAACTATGCGATGGGTTCTCATGTGATGCAATATGGTGATACCAAC  960
T  S  N  F  N  N  Y  A  M  G  S  H  V  M  Q  Y  G  D  T  N    320
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I  T  A  E  K  L  Y  L  Y  Q  G  F  D  P  A  T  V  N  F  P    340
CCACAAAACGGCAGGCTAGAAACTAAAATGGAAGTTGTTAACCAAAGAGATGCAGAACTT 1080
P  Q  N  G  R  L  E  T  K  M  E  V  V  N  Q  R  D  A  E  L    360
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F  L  L  W  Q  M  Y  Q  R  S  M  N  Q  S  E  N  K  T  D  I    380
CTCAAACAAATTGCGGAGACAGTGAAGCATAGGAAACACATAGATGGTAGCGTGGAATTG 1200
L  K  Q  I  A  E  T  V  K  H  R  K  H  I  D  G  S  V  E  L    400
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I  G  V  L  L  Y  G  P  G  K  G  S  S  V  L  Q  S  V  R  A    420
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P  G  S  S  L  V  D  D  W  T  C  L  K  S  M  V  R  V  F  E    440
ACTCACTGTGGGCACACTGACTCAGTATGGCATGAAACACATGCGAGCATTCGCCAACATT 1380
T  H  C  G  T  L  T  Q  Y  G  M  K  H  M  R  A  F  A  N  I    460
TGCAACAGTGGCGTTTCTGAGGCCTCCATGGAAGAGGCTTGTTTGGCAGCCTGTGAAGGC 1440
C  N  S  G  V  S  E  A  S  M  E  E  A  C  L  A  A  C  E  G    480
TACAATGCTGGGCTATTCCATCCATCAAACAGAGGCTACAGTGCTctgattttgggttttg 1500
Y  N  A  G  L  F  H  P  S  N  R  G  Y  S  A  ***               495
tacacaaaagctttaaagcccggttgatgatgtaatatttctctattgcattctgcctac  1560
tggtttctgctgcttgtgtcaaatttttctctaaactagagtagcccaatagcatacgtgt  1620
tatgtgcattggtcatgtatacaagtgtaatactaatacctttcctacataatataagatt  1680
agttagtttacttgtc poly(A)                                       1696
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Fig. 1 Nucleotide and deduced amino acid sequences of sVPE cDNA for the soybean homologue of a vacuolar processing enzyme. Stars indicate termination codons. The nucleotide sequence data will appear in the GSDB, DDBJ, EMBL, NCBI nucleotide sequence databases with the accession number D28876.

sor from soybean has a hydrophobic sequence which resembles that of a putative signal peptide at its N-terminus. Applying the method reported by von Heijne (1986), we predicted the cleavage site of the signal peptide to be on the C-terminal side of alanine-32 (indicated by a closed triangle in Fig. 2). Recently, Abe et al. (1993) reported an N-terminal 25-amino-acid sequence of an asparaginyl endopeptidase from jack bean. That sequence is identical to the sequence from glutamic acid-56 to valine-80 of the soybean protein (underlined in Fig. 2). By analogy to the jack bean enzyme, the N-terminal amino acid of the soybean enzyme may be glutamic acid-56, suggesting that the 23-amino-acid region from arginine-33 to aspartic acid-55 is a propeptide region that must be removed post-translationally (Fig. 2).

There is very low sequence homology between each signal-peptide region and between each N-terminal propeptide among the four enzymes compared here (Fig. 2). By contrast, the N-terminal regions of the putative mature enzymes are highly conserved. It is well known that both cysteine and histidine residues are required for the proteolytic activity of cysteine proteinases (Husain and Lowe 1970). Cysteine-81 and histidine-178 are found in the highly conserved regions (indicated by asterisks in Fig. 2) and they may have a crucial role in the enzymatic activity.

The existence of the mRNA for the homologue of the vacuolar processing enzyme suggests that a processing mechanism similar to that in castor bean is operative in soybean. Previously we detected vacuolar processing activity in soybean (Hiraiwa et al. 1993). To examine the existence of a protein related to the castor bean enzyme in soybean seeds, we conducted an immunoblotting analysis using a specific antibody against the enzyme from castor bean, as shown in Figure 3. We isolated protein bodies from soybean and castor bean seeds and the matrix fraction was prepared from each. Each matrix fraction, containing 0.4 munit of vacuolar processing activity, was subjected to SDS-PAGE and subsequent staining with Coomassie blue (Fig. 3, lanes 1 and 2) and immunoblotting analysis. A 39-kDa immunopositive band was detected in the soybean fraction (Fig. 3, lane 4), while a major band of a 37-kDa protein and a faint band of a 43-kDa protein were detected in

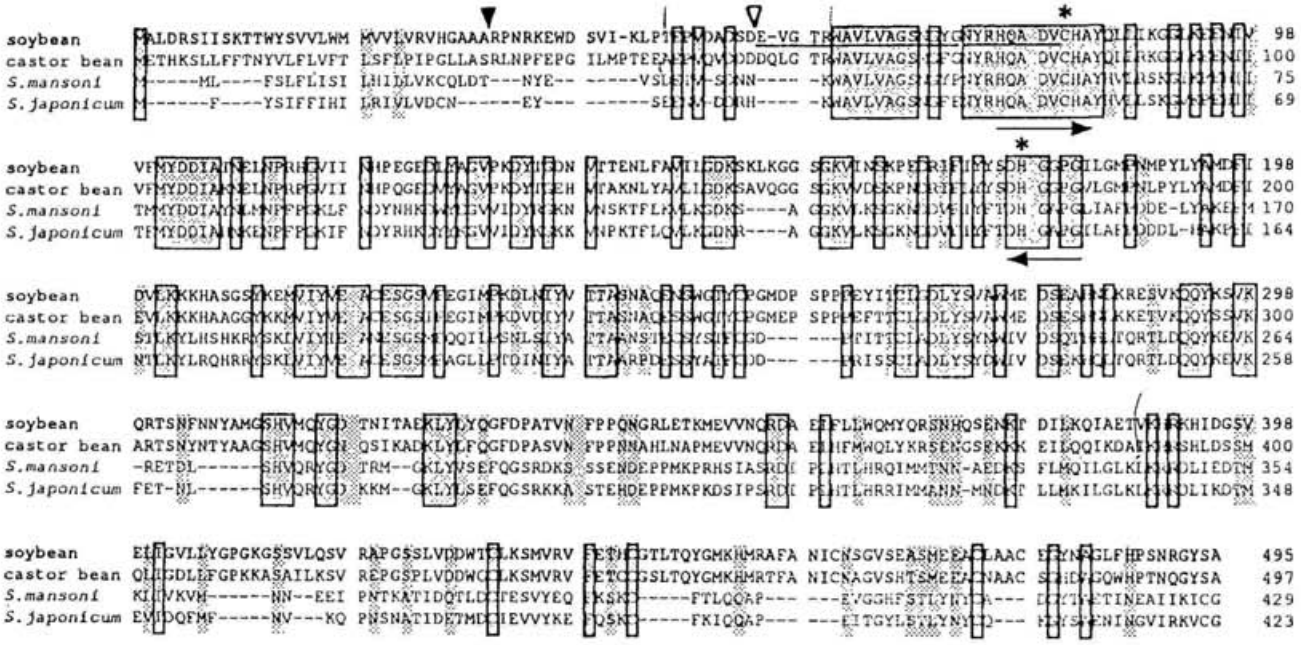

Fig. 2 Comparison of the sVPE-encoded protein with the vacuolar processing enzyme of castor bean and the putative cysteine proteinases of *S. mansoni* and *S. japonicum*. The primary sequences of the sVPE-encoded protein, the castor bean enzyme (Hara-Nishimura et al. 1993b), the putative cysteine proteinase of *S. mansoni* (Klinkert et al. 1989) and the putative cysteine proteinase of *S. japonicum* (accession number S31908 in the peptide sequence database of PIR) were aligned using the GeneWorks program (IntelliGenetics, Mountain View, CA, U.S.A.). Boxed residues indicate identical amino acids, and shaded residues indicate homologous amino acids. The N-terminal 25-amino-acid sequence of asparaginyl endopeptidase of jack bean reported by Abe et al. (1993) is underlined. The closed triangle indicates the putative site of cleavage of the signal peptide and the open triangle indicates the putative site of post-translational cleavage of the N-terminal propeptide. Asterisks indicate the cysteine and histidine residues at the possible active sites of the vacuolar processing enzymes. The two primers for screening of a cDNA library were synthesized on the basis of the sequences indicated by underlining with arrowheads.

the castor bean fraction (Fig. 3, lane 3). The minor 43-kDa protein is related to the castor bean enzyme, as described previously (Hiraiwa et al. 1993). These findings indicate that a protein that is immunologically related to the vacuolar processing enzyme of castor bean is localized in the matrix fraction of protein bodies of soybean seeds. Thus, it is possible that a similar processing mechanism to that in castor bean plays a role in processing of proproteins of soybean seed proteins.

To clarify whether the 39-kDa immunopositive protein of soybean has vacuolar processing activity, we separated the protein in the protein-body matrix of soybean by column chromatography on hydroxyapatite, as shown in Figure 4. Each fraction after chromatography was subjected to immunoblotting analysis and an assay of vacuolar processing activity. The processing activity with a synthetic decapeptide as substrate, whose sequence was derived from the sequence around a processing site of proglobulin, was bound to the column and eluted in peak of fractions 9–11 (Fig. 4C). The 39-kDa immunopositive protein was eluted in parallel with the elution profile of the processing activity

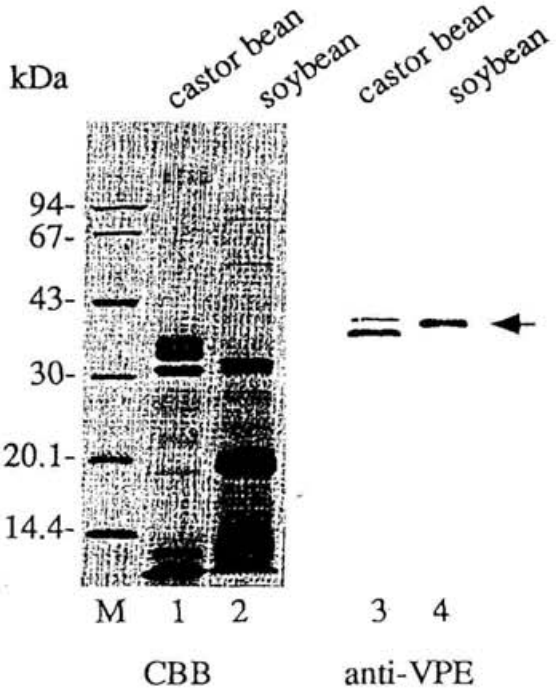

Fig. 3 Detection of vacuolar processing enzymes in the matrix of proteins bodies from castor bean and soybean. Protein bodies were isolated from dry seeds of castor bean and soybean by the glycerol method. Soluble matrix fractions were separated from insoluble crystalloids. Each matrix fraction, containing 0.4 munit of vacuolar processing activity, was subjected to SDS-PAGE with subsequent staining with Coomassie blue (CBB; lanes 1 and 2) or immunoblotting analysis (anti-VPE; lanes 3 and 4). The antibody used was raised against a fusion protein composed of a maltose-binding protein and a vacuolar processing enzyme from castor bean that had been expressed in *E. coli*. The molecular mass of each marker protein (lane M) is given on the left in kDa.

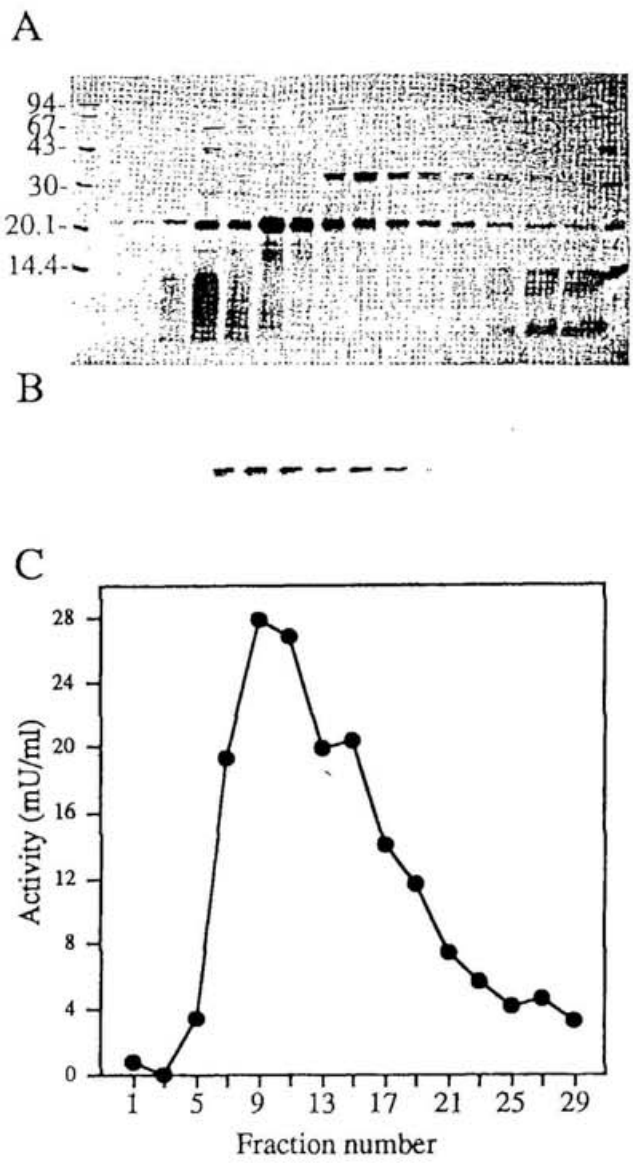

Fig. 4 Co-elution of vacuolar processing activity and an immunoreactive 39-kDa soybean enzyme from a hydroxyapatite column. Matrix proteins of soybean protein bodies were applied to a hydroxyapatite column and the proteins were eluted with a gradient of 0.01 M to 0.5 M sodium phosphate buffer, pH 6.0. (A) Proteins in each fraction after SDS-PAGE were stained with Coomassie blue. The molecular mass of each marker protein is given on the left in kDa. (B) An immunoblot of each fraction was obtained with the same antibody against the castor bean enzyme as described in the legend to Figure 3. (C) Vacuolar processing activity in each fraction was quantitated with a synthetic decapeptide as substrate.

(Fig. 4B). This result strongly suggests that the 39-kDa protein is responsible for the vacuolar processing activity.

We further examined the ability of the proprotein-processing activity to generate mature protein, as shown in Figure 5. Fractions 9 to 11 from the hydroxyapatite column were concentrated and used as an enzyme fraction. Dense vesicles from pulse-labeled endosperm of maturing castor bean seeds contained labeled proproteins that included proglobulin (Fig. 5, lane 1), which was used as a substrate for the processing enzyme. During the incubation of the proproteins with 0.5 munit of the soybean enzyme for 60 min, the proglobulin was converted to the mature globulin (Fig. 5, lane 2). Vacuoles isolated from the labeled endosperm of maturing castor bean seeds contained both the proglobulin and mature 11S globulin and these were used as size markers in the analysis (Fig. 5, lane 4). In the presence of 1 mM *N*-ethylmaleimide, the processing of the proglobulin was inhibited (Fig. 5, lane 3), indicating that the soybean processing enzyme is a cysteine proteinase as is the castor bean enzyme. The enzyme can process not only castor bean proglobulin but also pumpkin proglobulin to yield the corresponding mature forms (data not shown). This result suggests that the soybean enzyme has the ability to convert proglycinin to the mature form and that a processing mechanism similar to that in castor bean may function in the protein-storage vacuoles of soybean seeds.

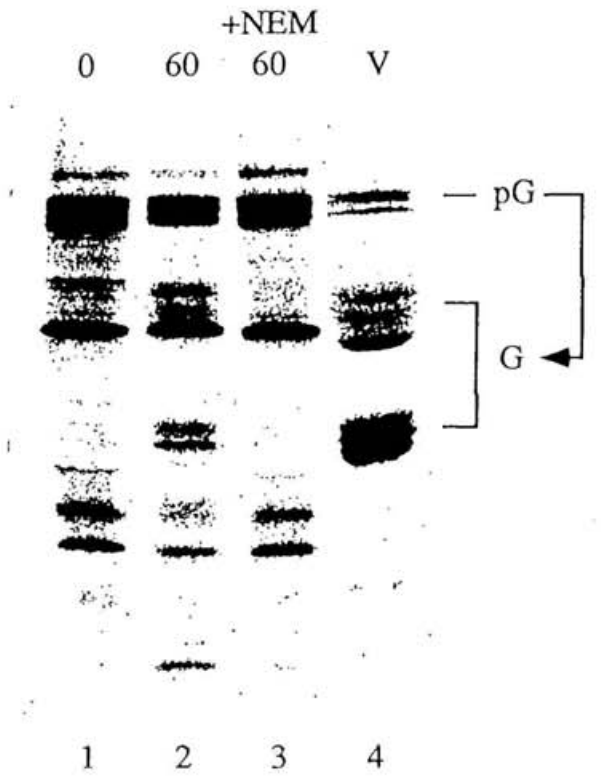

Fig. 5 Proteolytic processing in vitro of ^{35}S-labeled proglobulin to mature globulin by the purified enzyme from soybean. The labeled proproteins were located in the dense vesicles of the pulse-labeled endosperm of maturing castor bean seeds. Fractions 9 to 11 from the hydroxyapatite column, as shown in Figure 5, were pooled and used as purified enzyme. The labeled proproteins were incubated with 0.5 munit of the purified enzyme for 0 min (lane 1), for 60 min (lane 2), and for 60 min in the presence of *N*-ethylmaleimide (lane 3) at 37°C. After SDS-PAGE of each reaction mixture, the labeled proteins were visualized by fluorography. Vacuoles contained both the proglobulin (pG) and the mature 11S globulin (G), and these proteins were used as size markers in the fluorograph (lane 4). An arrow indicates the conversion of proglobulin to 11S globulin.

Scott et al. (1992) purified a proglycinin-processing activity from maturing soybean seeds and found associated molecular masses of 85, 65 and 23 kDa by SDS-PAGE. They suggested that both the 65-kDa and 23-kDa species were the products of degradation of an 85-kDa molecule. The purification of the enzyme was, however, only partial and their purified proteins appear to have been unrelated to a processing enzyme, as discussed by Muramatsu and Fukazawa (1993). The investigation by Muramatsu and Fukazawa (1993) showed that cysteine proteinases that convert proglycinin to the mature form could be purified from dry soybean seeds and they were designated collectively legumaturain. The molecular masses of 33 to 33.8 kDa of legumaturain were 5.2 to 6 kDa smaller than the 39 kDa of the vacuolar processing enzyme of soybean that we observed (Fig. 3). Recently Muramatsu and Fukazawa isolated a 1,332-bp cDNA for one of their isozymes of legumaturain (Abstract for Annual Meeting of the Molecular Biology Society of Japan, 1993). The deduced protein of 341 amino acids was shorter by 154 amino acids than sVPE-encoded protein, an indication that the sVPE cDNA clone that we isolated here encodes a different polypeptide from legumaturain. In addition, the specific activity of their purified legumaturain seemed to be very low; they used 8 μg of purified enzyme to cleave 50 μg of proglycinin within 13 h (Muramatsu and Fukazawa 1993). By contrast, under similar conditions to theirs, 0.06 μg of our vacuolar processing enzyme of castor bean was sufficient to process 10 μg of proprotein completely within 30 min (Hara-Nishimura et al. 1991). These results suggest that two different enzymes, our vacuolar processing enzyme and legumaturain, are responsible for processing of proglycinin in soybean.

The vacuolar processing enzyme plays a crucial role in the determination of the final structure of many seed proteins at the last step in their biosynthesis and regulates the biogenesis of protein-storage vacuoles in seeds. Previously, we detected the vacuolar processing activities in various plant tissues, including mature leaves, roots and hypocotyls (Hiraiwa et al. 1993). Thus, similar processing enzymes operate in plant vacuoles of a variety of tissues and

vacuolar processing appears to be a universal event in plant cells. Further analysis of the vacuolar processing mechanism is necessary to elucidate the physiological function of the processing of proproteins in vacuoles.

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Vacuolar Processing Enzyme Responsible for Maturation of Seed Proteins

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Summary

A vacuolar processing enzyme responsible for maturation of seed proteins was isolated from the castor bean and soybean. The processing enzyme belongs to a novel cysteine proteinase with a molecular mass of 37 kDa for castor bean and 39 kDa for soybean. The enzyme splits a peptide bond on the C-terminal side of an exposed asparagine residue of the proprotein precursors to produce their mature seed proteins such as 11S globulin and 2S albumin. Immunocytochemical localization of the enzyme in the vacuolar matrix of maturing castor bean endosperm indicates that the maturation of the seed proteins occurs in the vacuoles. Molecular characterization revealed that the enzyme is synthesized as an inactive precursor with a larger molecular mass. The results of immunoelectron microscopic analysis suggested that the precursor is transported to vacuoles *via* dense vesicles together with proproteins of seed proteins. After arriving at the vacuoles, the inactive precursor is converted into an active enzyme. This suggests that a cascade for proprotein processing is involved in the maturation of seed proteins. Vacuolar processing enzyme activity was found in various plant tissues and several cDNA homologues of the enzyme were isolated from different plants. Thus a similar processing enzyme is widely distributed in plant tissues and plays a crucial role in the maturation of a variety of proteins in plant vacuoles.

Key words: Castor bean (Ricinus communis), 2S albumin, 11S globulin, asparaginyl endopeptidase, cysteine proteinase, proprotein processing, protein body, seed protein, vacuolar processing enzyme.

Abbreviations: ER = endoplasmic reticulum; HPLC = high performance liquid chromatography; VPE = vacuolar processing enzyme.

Introduction

Proproteins of various seed proteins including 11S globulin, 2S albumin and lectins are well known to be post-translationally processed into mature proteins. However, the processing mechanism in plant vacuoles is very obscure. Several groups have investigated processing enzymes that convert proglobulin into 11S globulin in maturing seeds of castor bean (*Ricinus communis:* Hara-Nishimura et al. 1991, 1993 b; Hiraiwa et al. 1993), soybean (*Glycine max:* Muramatsu et al. 1994; Scott et al. 1992; Shimada et al. 1994) and pumpkin (*Cucurbita* sp.: Hara-Nishimura and Nishimura 1987), and that converts proricin into ricin in the castor bean (Harley and Lord 1985). A cDNA of one of the processing enzymes was isolated from the castor bean (Hara-Nishimura et al. 1993 b). The deduced primary sequence is 33 % identical in amino acid sequence to a putative cysteine proteinase of *Schistosoma mansoni* (Klinkert et al. 1989). The enzyme is an asparaginyl endopeptidase that belongs to a novel type of cysteine proteinases. The processing enzyme activity can be

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Fig. 1: The sequences around post-translational processing sites for seed proteins and vacuolar proteins from leaves and stigma. Proprotein processing occurs on the C-terminal side of an asparagine residue that is indicated by an asterisk. The processing site was determined by aligning the deduced precursor sequences with the N-terminal sequences of the respective mature proteins. Literature cited and accession number in sequence databases are as follows: 1) Hara-Nishimura et al. 1993a, D16560; 2) Sharief and Li 1982, S11500; 3) Krebbers et al. 1988, JA0161; 4) Ericson et al. 1986, Monsalve et al. 1991, A01329; 5) Altenbach et al. 1992, Ampe et al. 1986, S14946; 6) Allen et al. 1987, S01062; 7) Hayashi et al. 1988b, M36407; 8) Lycett et al. 1984, A22866; 9) Gatehouse et al. 1988, S00336; 10) Vonder Harr et al. 1988, JA0089; 11) Negoro et al. 1985, A23497; 12) Staswick et al. 1984, A91341; 13) Hirano et al. 1984, A92524; 14) Hirano et al. 1985, A91145; 15) Shotwell et al. 1988, JA0160; 16) Gatehouse et al. 1982, A03344; 17) Araki and Funatsu 1985, A24041; 18) Roberts et al. 1985, A24261; 19) Funatsu et al. 1988; S32429; 20) Bowles et al. 1986, A03357; 21) Wu and Lin 1993, JH0607; 22) Santino et al. 1992, A27350; 23) Gatehouse et al. 1987, Y00440; 24) Wilkins and Raikhel 1989, JQ1102; 25) Graham et al. 1985, A24048; 26) Atkinson et al. 1993.

found not only in seeds but also in non-storage tissues such as hypocotyls, roots and mature leaves (Hiraiwa et al. 1993). Thus, we designated the enzyme as a vacuolar processing enzyme (VPE).

Here we discuss about the processing mechanism in plant vacuoles and the molecular characterization of the VPEs.

Vacuolar processing enzyme converts different proproteins into their respective mature forms

Figure 1 shows the 20-amino acid sequences around the post-translational processing sites for seed proteins and vacuolar proteins. All the processing sites were experimentally determined by aligning the deduced precursor sequences with the N-terminal sequences of the respective mature proteins. Various seed proteins including 2S albumins, 11S globulins, vicilins, toxins, lectins, proteinase inhibitors and α-amylase inhibitors are produced after a proteolytic cleavage at the C-terminal side of an asparagine residue of their proprotein precursors. One question arises here; whether a single enzyme is responsible for processing many proteins with a large variability of molecular structure. If this is true, how can it recognize the numerous varieties of processing sites? Characterization of a purified processing enzyme is required to answer the question.

Conversion of proglobulin into mature 11S globulin occurs in the vacuoles and protein bodies of seeds of the castor bean and soybean. We used the isolated protein bodies as starting materials to purify the processing enzymes of castor bean (Hara-Nishimura et al. 1991) and soybean (Shimada et al. 1994). The purified enzymes of castor bean and soybean are 37 kDa and 39 kDa, respectively. Both enzymes can cleave a peptide bond on the C-terminal side of an asparagine residue of a synthetic decapeptide, the sequence of which is derived from that around the processing site of pumpkin proglobulin, precursor to 11S globulin.

To demonstrate that the purified enzyme can cleave the authentic proprotein precursors synthesized on the rough endoplasmic reticulum (rER) in the cells of maturing seeds, we performed *in vitro* processing using proproteins in the isolated dense vesicles and ER as substrates. The findings clearly show that the castor bean enzyme is capable of converting not only proglobulin but also pro2S albumin into their respective mature forms (Hara-Nishimura et al. 1991). This indicates that a single VPE can split a peptide bond at the C-terminal side of an asparagine residue of several proproteins to make mature seed proteins.

Abe et al. (1993) isolated an asparagine endopeptidase from the jack bean that exhibits strong structural homology to the castor bean enzyme (discussed below). They demonstrated that the jack bean enzyme could convert proconcanavalin A into mature concanavalin A. This is in agreement with the evidence that a VPE-like asparaginyl endopeptidase plays a role in maturation of various seed proteins as shown in Figure 1. However, no consensus sequences were found in the

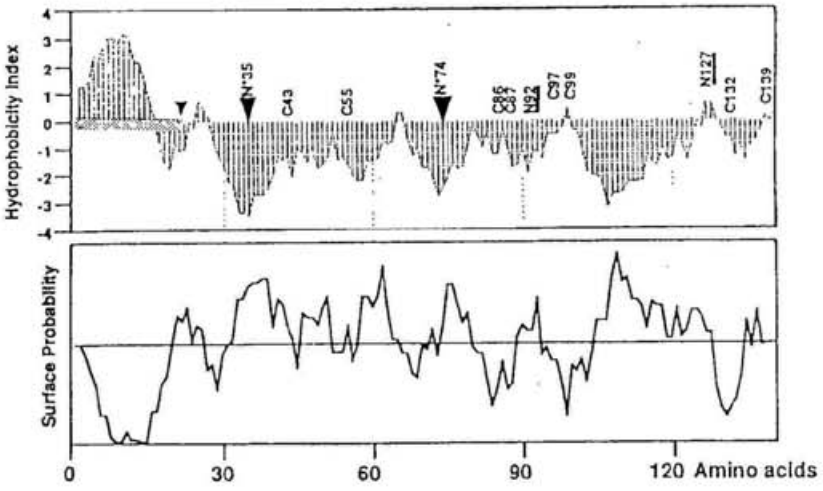

Fig. 2: Vacuolar processing occurs on the C-terminal side of two asparagine residues in hydrophilic regions of the pro2S albumin molecule. Analyses of hydropathy and surface probability of the primary sequence of prepro2S albumin were performed. The mean hydrophobicity index was computed according to the algorithm of Kyte and Doolittle (1982) with a window of 11 residues, and surface probability was according to the algorithm of Emini et al. (1985). The closed triangle indicates the cleavage site of the signal peptide. All asparagine and cysteine residues are indicated as N and C with their respective positions. The two arrowheads indicate the sites of vacuolar processing. Two asparagine residues 35 and 74 with asterisks are located at the vacuolar processing sites. Asparagine residues 92 and 127 are not involved in the processing.

sequences around processing sites. How can VPE recognize the processing sites of different proproteins with a large variability of molecular structure?

Vacuolar processing enzyme cleaves a peptide bond at the C-terminal side of an exposed asparagine residue on molecular surface of proproteins

To clarify the mechanism of processing by VPE in greater detail, we characterized the post-translational processing sites of pro2S albumins. The N-terminal amino acid sequence analysis of the two subunits of mature 2S albumin in pumpkin indicates that proteolytic cleavages occur on the C-terminal side of the asparagine residues at positions 35 and 74 of prepro2S albumin (Hara-Nishimura et al. 1993 a). The positions of the two asparagine residues have been conserved in the precursors to 2S albumins from various species of pumpkin, castor bean, *Arabidopsis* and rapeseed, with the exception of the sunflower and Brazil nut (discussed below), as shown in Figure 1. Proteolytic cleavage certainly occurred on the C-terminal side of the conserved asparagine residues of pro2S albumins in various plants.

Figure 2 shows the results of the hydropathy analysis and surface probability of the primary sequence of prepro2S albumin. Asparagine residues 35 and 74 are both located in extremely hydrophilic regions of the prepro2S albumin sequence. There are two more asparagine residues, at positions 92 and 127, in the prepro2S albumin sequence. Asparagine 127 is located in the most hydrophobic region of the mature 2S albumin sequence, and it seems likely that this residue is on the inside of the molecule. Asparagine 92 is close to four cysteine residues that may engage in the formation of disulfide bonds. The locations of the disulfide bonds are estimated from the location of such bonds in the α-amylase inhibitor from wheat (Maeda et al. 1983), which exhibits considerable similarity to 2S albumins. The environment around the asparagine residues 92 and 127 might prevent the VPE from having access to the adjacent peptide bonds. These findings suggest that VPE recognizes the two asparagine residues in the hydrophilic regions on the molecular surface. Bowles et al. (1986) reported that asparagine residues at post-translational cleavage sites of proconcanavalin A were also located on the molecular surface. These findings strongly suggest that VPE cleaves peptide bonds at the C-terminal sides of the exposed asparagine residue on the molecular surface of proprotein precursors.

Vacuolar processing enzyme is located in the matrix region of vacuoles of maturing seeds

Protein storage vacuoles of maturing seeds are composed of crystalloids, matrix and membrane. 2S albumin is located in the vacuolar matrix (Hara-Nishimura et al. 1993 a), and 11S globulin is the primary constituent of the vacuolar crys-

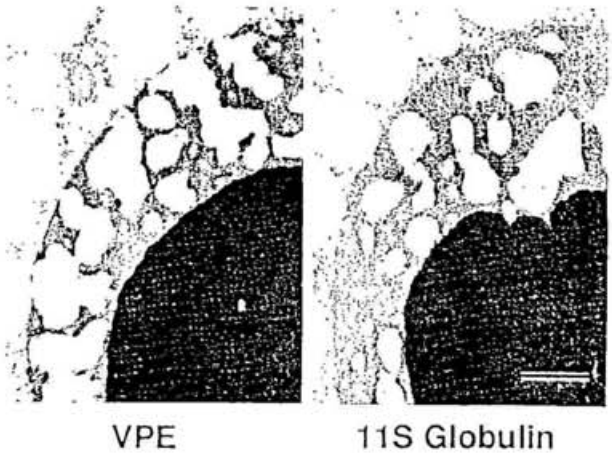

Fig. 3: Immunocytochemical localization of a vacuolar processing enzyme in maturing castor bean endosperm. Ultrathin section of maturing castor bean endosperm was incubated with either an anti-VPE antibody (left) or anti-11S globulin antibody (right). Bar = 1 μm.

talloid (Hara-Nishimura et al. 1982, 1987). To examine the subcellular location in which post-translational processing of proglobulin occurs, we performed an immunocytochemical analysis of maturing castor bean endosperm using a specific antibody against either the purified VPE or 11S globulin. Figure 3 (left) shows that VPE is localized to the electron-dense area of the matrix region in vacuoles of the maturing castor bean endosperm, but not in the vacuolar membrane or in the crystalloid (Fig. 3, left). By contrast, when using the specific antibody against 11S globulin, we found gold particles distributed throughout the electron-dense area of the matrix as well as the crystalloid in protein storage vacuoles (Fig. 3, right). Selective concentration of the VPE suggests that proglobulin processing occurs in the matrix region of vacuoles of maturing seeds.

Cell fractionation of pulse-chase-labeled cotyledons of maturing pumpkin seeds reveals that proglobulin and pro2S albumin also accumulated transiently in the vacuoles (Hara-Nishimura et al. 1985, 1993a; Hayashi et al. 1988a). The proglobulin and pro2S albumin that are newly imported into the vacuole are thought to be present in the vacuolar matrix and then converted into the respective mature forms by the action of VPE. Conversion of proglobulin into 11S globulin reduces the solubility of the protein (data not shown), causing formation of the crystalloid.

Proglobulin is converted into mature 11S globulin in the matrix fraction of vacuoles isolated from maturing pumpkin cotyledons (Hara-Nishimura and Nishimura 1987). The optimum pH of the castor bean VPE is the same as that of the vacuolar interior, about 5.5, as shown in Figure 4. The findings suggest that the post-translational processing of various proprotein precursors only occurs within a limited area of the vacuolar matrix.

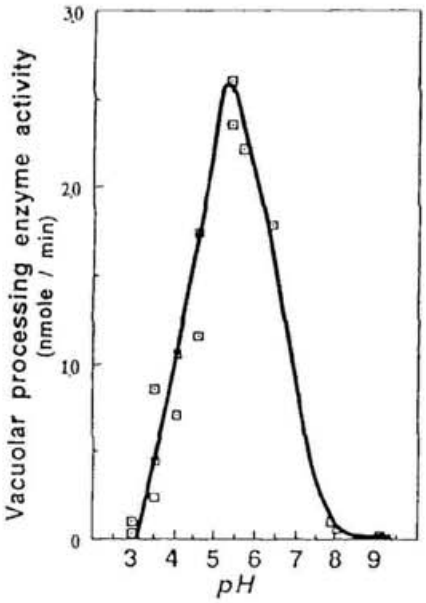

Fig. 4: Optimum pH of vacuolar processing enzyme purified from castor bean endosperm. Decapeptide whose sequence was derived from the sequence around the processing site of pumpkin proglobulin was used as substrate. A reaction mixture containing the purified enzyme and 5 nmole of the decapeptide substrate in 10 µL of 0.1 M dithiothreitol, 0.1 mM EDTA and 0.1 M various buffer solutions; Na-acetate buffer (pH 3.0 to 5.0), citrate-phosphate buffer (pH 5.5 to 7.0) and Tris-HCl buffer (pH 7.5 to 9.0). This was incubated for 1 to 30 min at 37 °C and followed by HPLC analysis.

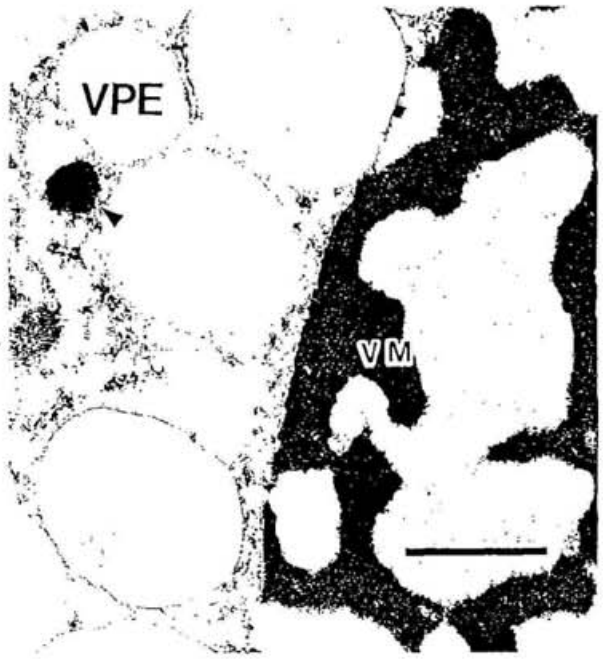

Fig. 5: Dense vesicles are involved in the transport of the vacuolar processing enzyme in maturing endosperm cell of castor bean. Ultrathin section of maturing castor bean endosperm was incubated with an anti-VPE antibody. Gold particles are detected in vacuolar matrix (VM) and dense vesicle that is indicated by an arrowhead. Bar = 1 µm.

Vacuolar processing enzyme is transported to vacuoles via dense vesicles

Major seed proteins, 11S globulin and 2S albumin, are transported from ER to vacuoles *via* dense vesicles in the cells of maturing seeds of pumpkin and castor bean (Akazawa and Hara-Nishimura 1985; Fukasawa et al. 1988; Hara-Nishimura et al. 1985; Hara-Nishimura et al. 1993a). The dense vesicles isolated from maturing pumpkin cotyledons contain high levels of a variety of proproteins (Hara-Nishimura et al. 1991). Immunocytochemical studies revealed that VPE was selectively localized in the dense vesicles that mediate transport of proproteins of various seed proteins to vacuoles, as Figure 5 shows. This indicates that VPE is transported to vacuoles *via* dense vesicles together with proprotein substrates, such as proglobulin and pro2S albumin. However, endogenous maturation of seed proteins does not occur in these vesicles (Hara-Nishimura et al. 1985). Thus, VPE in the prevacuolar compartment must be a latent form

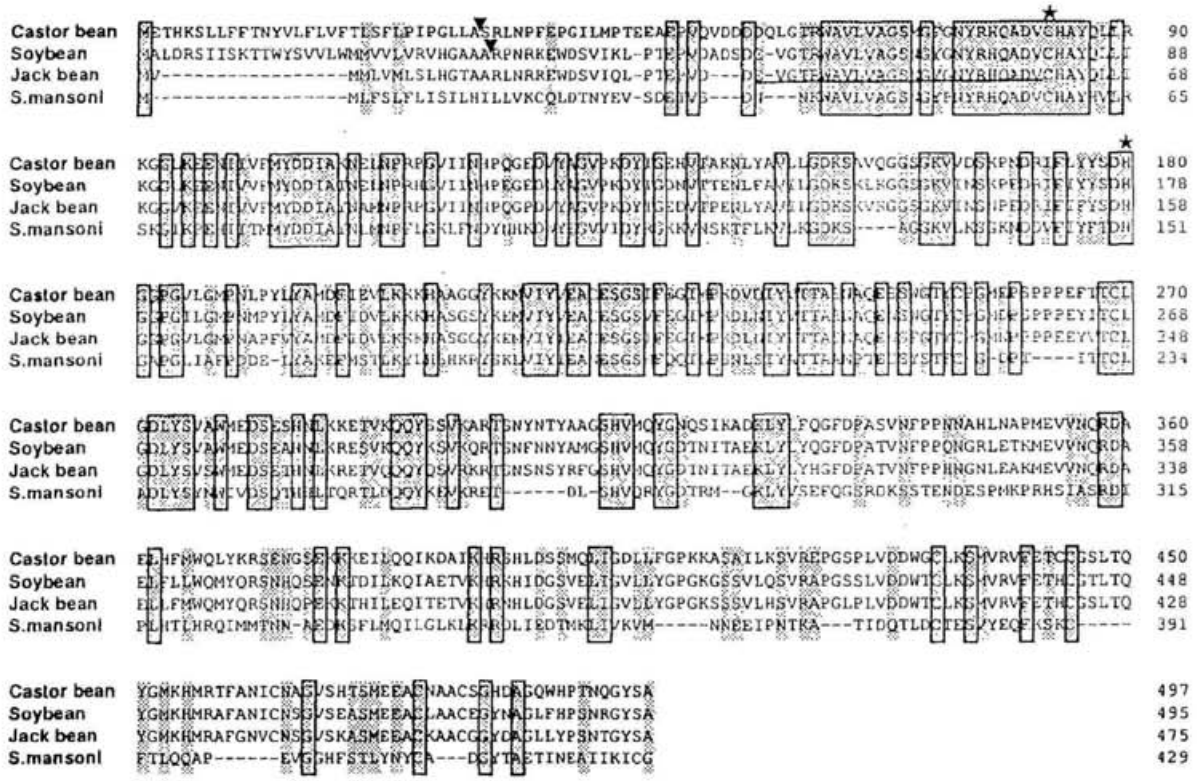

Fig. 6: Comparison of vacuolar processing enzymes of castor bean and soybean with jack bean legumain and a putative cysteine proteinase of *S. mansoni*. The primary sequences of castor bean VPE (Hara-Nishimura et al. 1993 b), soybean VPE (Shimada et al. 1994), jack bean legumain (Takeda et al. 1994) and a putative cysteine proteinase of *S. mansoni* (Klinkert et al. 1989) are aligned using a program of GeneWorks (IntelliGenetics, Mountain View, CA, USA). Boxed residues indicate identical amino acids, and shaded residues indicate homologous amino acids. The closed triangle indicates the putative cleavage site of the signal peptide of castor bean VPE and soybean VPE. An N-terminal sequence of jack bean legumain is underlined, while an N-terminal amino acid of castor bean VPE is blocked. Cysteine and histidine that are found in the highly conserved regions (indicated by asterisks) may be a possible active site of the enzyme.

that the enzyme must be activated in vacuoles, as Figure 7 shows. To clarify the mechanism of vacuolar processing essential for biogenesis of protein storage vacuoles in seeds, we examined the molecular structure and activation of the VPE.

Molecular structure of vacuolar processing enzyme

We isolated cDNAs for VPEs of castor bean (Hara-Nishimura et al. 1993b) and soybean (Shimada et al. 1994). Both cDNAs encode 55-kDa precursors. The deduced precursor to castor bean VPE is 71% identical to that of soybean, 70% to an asparaginyl endopeptidase of jack bean (Takeda et al. 1994) and 33% to a putative cysteine proteinase of the human parasite *Schistosoma mansoni* (Klinkert et al. 1989), as Figure 6 shows. The VPEs of castor bean (Hara-Nishimura et al. 1991), soybean (Shimada et al. 1994) and pumpkin (Hara-Nishimura and Nishimura 1987) are inhibited by a thiol reagent such as N-ethylmaleimide, monoiodic acetic acid and p-chloromercuribenzoate, an indication that the enzyme is a cysteine proteinase. However, the sequences of VPEs do not exhibit significant homology to known cysteine proteinases, including papain from papaya and mammalian cathepsins. This suggests that VPEs and *Schistosoma* enzyme belong to a novel type of cysteine proteinase.

The 55-kDa precursors to VPE are much larger than the 37-kDa and 39-kDa active enzymes purified from castor bean and soybean, respectively. Immunoblotting using the crude extract of these seeds demonstrates that the 37-kDa and 39-kDa enzymes are actual mature enzymes and are not degradation products during purification. Hydropathy analysis shows that each precursor has a hydrophobic sequence of a putative signal peptide at the N terminus. According to the method reported by von Heijne (1986), the cleavage site of the signal peptide is predicted to be at the C-terminal side of alanine 31 of the castor bean enzyme and alanine 32 of the soybean enzyme, respectively (indicated by a closed triangle in Figure 6).

Because the N-terminal amino acid of the mature VPEs of castor bean is blocked, it remains to be solved whether a small prosequence fragment between a signal peptide and the mature enzyme domain is present. Recently, the N-terminal amino acid of jack bean enzyme was reported to be glutamic

acid-36 (Takeda et al. 1994). From the analogy with the jack bean enzyme, the N-terminal amino acid may be aspartic acid-57 for castor bean enzyme and glutamic acid-56 of soybean enzyme. This suggests that the 24-amino acid region from serine-32 to aspartic acid-56 of castor bean VPE and 23-amino acid region from arginine-33 to aspartic acid-55 of soybean VPE are propeptide regions to be removed post-translationally (Fig. 6). Overall the findings suggest that the precursor is composed of a signal peptide, an N-terminal propeptide fragment, an active processing enzyme domain, and a C-terminal propeptide fragment.

A latent precursor to vacuolar processing enzyme is activated in vacuoles

The presence of a signal peptide at the N terminus indicates that the VPE is synthesized on the ER, and after cleavage of the signal peptide the 55-kDa precursor is converted into a 51-kDa precursor that is transported to the vacuoles. The result of an *in vitro* vacuolar processing experiment of the transformed cells shows that the larger precursor has no vacuolar processing activity. The functional expression suggests that the activation of the VPE requires proteolytic cleavage of the propeptide fragment of the precursor (Hara-Nishimura et al. 1993 b). These findings demonstrate that the precursor of the VPE in the dense vesicles is a latent form, which is transported to vacuoles and then activated in the vacuolar matrix by proteolytic cleavage of propeptide fragments. This strongly suggests that a cascade of processing plays a crucial role in the maturation of seed proteins in protein storage vacuoles of maturing seeds, as shown in Figure 7.

Although there is a very low sequence homology in each region of the signal peptides and the N-terminal propeptides among the four enzymes, the N-terminal region of the mature enzyme is highly conserved (Fig. 6). It is well known that both cysteine and histidine residues are required for the proteolytic activity of cysteine proteinases (Husain and Lowe 1970). Cysteine and histidine that are found in the highly conserved regions (indicated by asterisks in Figure 6) may be an active site of the enzyme. By contrast, the C-terminal region of ~100 amino acids of castor bean and soybean enzymes is less homologous. Thus, it is most likely that the region is not essential for processing activity. As described above, VPE recognizes exposed asparagine residues on the molecular surface of proproteins. Asparagine-374 of castor bean VPE precursor is also located in the most hydrophilic region. Autolysis of the C-terminal side of the asparagine residue may convert an inactive precursor into a 37-kDa active form of castor bean. Alternatively, the most hydrophilic region might be exposed on the molecular surface of the VPE precursor, and some proteolytic enzyme may easily attack the region. Neurath (1989) reported that the sites of proteolytic processing are generally in relatively flexible interdomain segments or surface loops. It is likely that proteolysis and/or autolysis induces a conformational change in the inactive precursor and then generates an active VPE *in vivo*.

The N terminus of the mature 32-kDa *Schistosoma* enzyme is located at valine-32 of the 49-kDa precursor (Klinkert et al. 1989). This indicates that a C-terminal fragment of ~ 14 kDa is post-translationally removed from the precursor to produce the mature enzyme. Götz and Klinkert (1993) reported that insect cells expressing the precursor of the *Schistosoma* enzyme had no proteolytic activity on hemoglobin. These observations are consistent with the results showing that the VPE precursor expressed in *E. coli* had no processing activity and that removal of the propeptide fragment converts the inactive precursor into an active VPE.

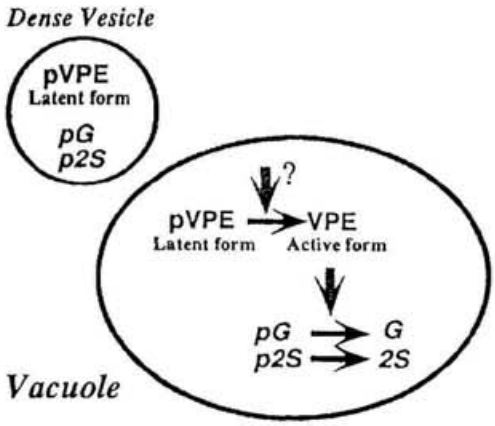

Fig. 7: Hypothetical model for an intracellular transport and activation of a vacuolar processing enzyme in maturing endosperm cells of castor bean. A latent precursor of castor bean VPE is transported from the endoplasmic reticulum to a vacuole *via* dense vesicle along with proproteins of seed proteins. The latent VPE precursor is converted into an active form by removal of the propeptide in the vacuole. The resulting active VPE catalyzes maturation of various seed proteins including the 11S globulin and 2S albumin.

Another proteinase may be involved in the post-translational modification of seed proteins

Sun et al. (1987) suggested that a 15-kDa pro2S albumin of Brazil nut is rapidly processed to a 12-kDa intermediate, which accumulates in the developing seed for more than 2 months before it is further processed to the mature form. The Brazil nut 2S albumin conserves asparagine-36 at the first processing site in the precursor sequence, but lacks an asparagine residue at the second processing site (Figs. 1, 2). This suggests that although the VPE can cleave a peptide bond on the C-terminal side of asparagine-36 to make a 12-kDa intermediate, it cannot split the intermediate into the two subunits of the mature form. Thus another proteolytic enzyme can be involved in the second processing of the 12-kDa intermediate to make two subunits of the mature 2S albumin. Accumulation of a 12-kDa intermediate in the developing Brazil nut seeds indicates that the second processing is a limiting step of maturation of the 2S albumin.

The pro2S albumins of castor bean, Brazil nut, *Arabidopsis* and rapeseed contain a linker peptide between the two subunits of the mature protein. The linker peptide might be cleaved off by another endopeptidase or carboxypeptidase. Since one amino acid at the C-terminus of rapeseed pro2S

albumins is removed, a carboxypeptidase must exist in the vacuoles of the developing seeds. Pulse-chase experiment of developing pumpkin cotyledons showed that the labeled proglobulin and pro2S albumin were transiently detected in the vacuoles, but did not accumulate in the vacuoles (Hara-Nishimura et al. 1985; Hara-Nishimura et al. 1993 a). This indicates that these proproteins are converted into their mature forms by the action of VPE, immediately after the translocation into the vacuoles. The vacuolar processing might precede the cleavages of the linker peptides and the C-terminal peptides of proprotein precursors to seed proteins. When and how the further cleavage occur in the vacuoles remains to the solved.

Physiological role of vacuolar processing in plants

Vacuolar processing at asparagine residues of the *Phaseolus* α-amylase inhibitor proprotein is shown to be required for its activation (Pueyo et al. 1993, Fig. 1). Such proteolytic processing associated with the activation of protein function is a common mechanism of physiological regulation and is well characterized in the case of animal cells, including a cascade for blood coagulation and prohormone processing.

The VPE responsible for maturation of various proteins plays a crucial role in the biosynthesis of vacuolar components and regulates the biogenesis of protein-storage vacuoles in seeds. However, the vacuolar processing activity is detected not only in seeds but also in different non-storage tissues including roots, hypocotyls and leaves of castor bean (Fig. 8) and of other plants (Hiraiwa et al. 1993). The proprotein processing for proteinase inhibitors of tomato leaves (Graham et al. 1985) and *Nicotiana alata* stigma (Atkinson et al. 1993) occurs on the C-terminal side of an asparagine residue, as Figure 1 shows. Recently we found that membrane proteins, MP27 and MP32, of the pumpkin protein body are synthesized as a single precursor, which is processed at the C-terminal side of an asparagine residue to produce the two membrane proteins (Inoue et al. 1995). These findings suggest that the vacuolar processing is a universal event in vacuoles of a broad range of plant tissues and that a VPE similar to the enzyme we describe here functions on maturation of various vacuolar proteins.

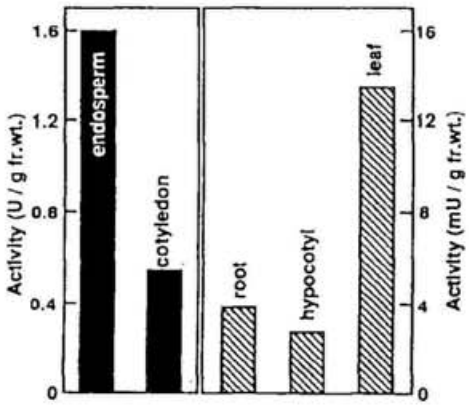

Fig. 8: Distribution of vacuolar processing enzyme in various tissues of castor bean. The VPE activity was assayed with a synthetic decapeptide as substrate and the products of the reaction were analyzed by capillary electrophoresis. One unit was defined as 1 μmole C-terminal pentapeptide produced per min.

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